Thesis for the degree
of Candidata Scientiarum

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Determination of catecholamines using large volume injection micro-liquid chromatography coupled to electrospray ionization-mass spectrometry (µLC-ESI-MS) – Hypercarb columns and their limitations

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Nihil tam difficile est
quin quaerendo investigani posit
(Publius Terentius Afer, 190-159 BC)

(Nothing is so difficult, that it cannot be investigated)

CONTENTS

PREFACE

This work has been carried out at the University of Oslo, Faculty of Mathematics and Natural Science, Department of Chemistry from January 2003 to August 2004. Ph.D. Anders Holm, Professor Elsa Lundanes and Professor Tyge Greibrokk were my supervisors. Parts of the theoretical education for the degree were taken at the Universität Leipzig, Germany and the Friedrich Schiller Universität Jena, Germany.

During my work I attended the 16. Norwegian Symposium of Chromatography in Sandefjord, January 2004 and presented the preliminary work on a poster. A copy of the poster is attached in the appendix.

This work deals with both the development of a µLC-ESI-MS method for the determination of catecholamines in brain samples and the limitations of the Hypercarb stationary phase used in the method. Due to problems with oxidation of both the stationary phase as well as the analytes considerable time was spent to develop the method. Efforts were focused upon suppressing or preventing of oxidation to get a repeatable method. Therefore, analysis of real samples is not included in this study.

I would like to thank my supervisors for giving me an interesting and challenging problem to deal with in my thesis. I also would like to thank them for their excellent guidance through this work, for the interesting discussions I have had with them and their moral support when I got stuck in a problem.

I would like to thank my fellow students for their help and guidance, and for the social life that we had in our group both at school and in our free time. Especially I would like to thank Aase Marit S. Ramton for the time we spent together in Germany and Espen Storbråten for the interesting discussions we had and his help with numerous PC questions.

Thanks to Hege Lynne and her staff at the analytical course laboratory for their help with equipment and for always making me feel welcome and comfortable.

Finally I would like to thank my family for their love and support. Especially I would like to thank my parents for making it possible for me to study in Norway. They were always a great inspiration and I had some good conversations with them about my work. Thanks as well for the comments and corrections.

At the end I would like to thank Trond for his love and patience with me through this time. I would also like to thank him for his professional help with the AutoCAD figures.
Thank you for being by my side!

Oslo, Norway, October 2004

Sandra Rinne

ABBREVIATIONS

AcN	acetonitrile
AcOH	acetic acid
CE	capillary electrophoresis
cLOD	concentration limit of detection
cLOQ	concentration limit of quantification
D	dopamine
E	epinephrine
EC	electrochemical (detector)
EIC	extracted ion chromatogram
ESI	electrospray ionization
FA	formic acid
HFBA	heptafluoroacetic acid
i.d.	inner diameter
LC	liquid chromatography
m/z	mass to charge ratio
MeOH	methanol
mLOD	mass limit of detection
mLOQ	mass limit of quantification
MP	mobile phase
MS	mass spectrometer / mass spectrometry
NE	norepinephrine
o.d.	outer diameter
PEEK	Poly-ether-ether-ketone
PFPA	pentafluoropropionic acid
PS-DVB	polystyrene-divinyl benzene
RP	reversed-phase
RSD	relative standard deviation
SE	serotonin
S/N	signal to noise ratio
SP	stationary phase
TDC	time to digital converter
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIC	total ion current
TOF	time of flight
UV	ultra violet
µ LC	micro liquid chromatography

ABSTRACT

The present work presents a fast and sensitive capillary liquid chromatography (μLC) column-switching method with electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) detection for the simultaneous determination of dopamine (D), epinephrine (E), norepinephrine (NE) and serotonin (SE). A sample volume of 100 μl was loaded on a 25 x 0.32 (i.d.) mm 5 μm Hypercarb column at a flow rate of 25 μl/min. The loading mobile phase contained 99.9% H_2O and 0.1% pentafluoropropionic acid (PFPA) as ion-pairing agent. A water–acetonitrile gradient containing 0.1% acetic acid (AcOH) in both reservoirs backflushed the compounds onto a 34 x 0.32 (i.d.) mm 5 μm Hypercarb analytical column with a flow rate of 5 μl/min. The analytes were separated in less than 5 min and the total time for one injection, including reconditioning was only 22 min. The analytes were detected in positive ion modus with the molecular ion masses $[M+H]^+$ of 154.1, 184.1, 170.1 and 177.1 for D, E, NE and SE, respectively. Additionally fragments of masses $[M+H-17]^+$ for D and SE and $[M+H-18]^+$ for E and NE were used for detection. The limit of detection (LOD) was 1.5 ng/ml, 0.75 ng/ml, 3.0 ng/ml and 1.5 ng/ml for D, E, NE and SE, respectively. The method was validated using a concentration range of 7.5–70 ng/ml for D, 3.0–70 ng/ml for E and 10–125 ng/ml for NE and SE. The calibration curves had R^2-values of 0.992, 0.997, 0.985 and 0.998 for D, E, NE and SE, respectively. The within-day (n = 3) and the between-day (n = 3) precision of the retention times were in the range of 0.7–5.9% and 1.2–3.7%, respectively. The within-day (n = 3) and the between-day (n = 3) precision of the peak areas was in the range of 4.8–59% and 8.0–72%, respectively.

Limitations of the stationary phase material and efforts to eliminate oxidation of both the stationary phase and the analytes are discussed.

1. Introduction

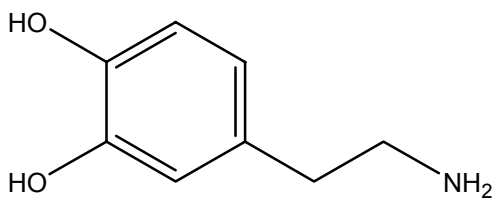
Neurons are nerve cells in the brain, muscle or gland. They receive and evaluate information from other neurons or receptor cells as well as pass on information to nerve cells. Neurons transmit information by nerve impulses, short breakdowns of the resting potential because of changes in the ion balance along their axons. At the ends of an axon these electrical signals result in the release of chemical messenger substances, the neurotransmitters. The neurotransmitters are transported through the synaptic gap to the dendrite of the next neuron where they interact with receptor sites. A change in the potential is induced starting a new signal along the second neuron. A detailed figure in the appendix shows the process (figure A10).

Neurotransmitters have important biological, pharmacological and physiological functions. Knowledge about neurotransmitters and their functions is crucial for the understanding of circuits in the brain. This helps finding circuits that are responsible for mental disorders as Parkinson and Alzheimer's disease, depression and schizophrenia and the biological basis of other mental illnesses. Determination of neurotransmitters helps learning about processes like memory storage, attention, sleep, movement, cognition, emotion, learning, hormone and blood pressure regulation [1].

1.1 Catecholamines

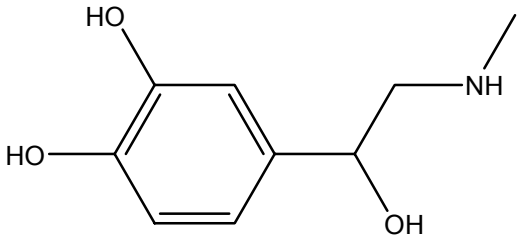
One important group of neurotransmitters are catechol- and indolamines (hereafter for simplification called catecholamines). The main sites of catecholamine production are the brain, the adrenal medulla and sympathetic neurons [2]. Structural formulas of four catecholamines used in this study are shown in figure 1. As can be seen from figure 1, catecholamines are small, polar molecules with basic pK_a–values. Catecholamines have a common structural element, the o-hydroxyphenol which has the trivial name catechol and gives them their names. The catechol ring is easily oxidized and catecholamines are light- and heat sensitive [2]. Thus, care has to be taken during preparation of solutions of catecholamines. Additives such as acetic acid [3] or hydrochloric acid [4] are added during preparation of stock solutions. Salts of the sulfurous acid as anti-oxidant can be used except

when mass spectrometry (MS) detection has been applied [5-7]. The substituted benzene ring gives catecholamines a natural ultra violet (UV) absorbance at about 220 nm.



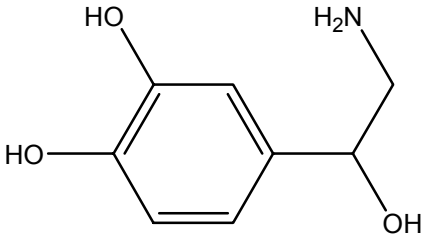
Dopamine (D)

pK_a = 9.41 & 9.99



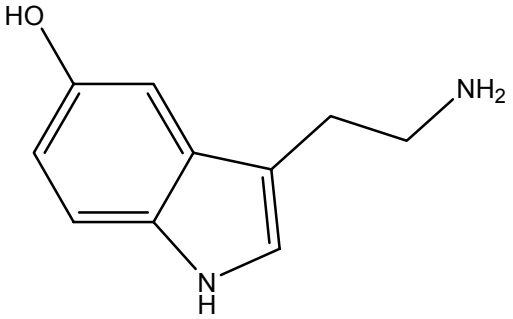
Epinephrine (E)

pK_a = 9.60 & 9.16



Norepinephrine (NE)

pK_a = 9.57 & 8.30



Serotonin (SE)

pK_a = 9.96 & 10.31

Figure 1: Structural formulas of the four catecholamines used in this study and their pK_a-values. Formulas: ChemDraw Ultra 8.0 (CambridgeSoft Corporation, Cambridge, MA, USA), pK_a-values: SciFinder Scolar 2004 (American Chemical Society, Washington, DC, USA).

1.2 Methods of determination

A review on the determination of catecholamines has recently been published by Peaston and Weinkove [2].

At acidic pH, separation techniques using ionic species, such as electrophoretic techniques are suitable. A capillary electrophoresis (CE) method coupled to electrospray ionization – mass spectrometry (ESI-MS) for the determination of catecholamines has been described by Peterson et al. [4, 8]. However, liquid chromatography (LC) has been the main technique for the determination of catecholamines using reversed-phase or ion-pairing as the main separation principle. Many different LC methods for determination have been described, an indication that none are ideal even with detection methods capable of low detection limits.

At acidic pH, separation principles using ionic interactions (ion-exchange chromatography) can be applied as well. Some articles using this separation principle have been published [9].

For the detection of catecholamines, several methods can be used. UV detection suffers from poor sensitivity and is not selective enough. To enhance sensitivity and selectivity, derivatization can be made to obtain fluorescent or chemiluminescent properties of the molecules. Numerous derivatization reagents and techniques have been reported [10-13]. Yoshitake and co-workers describe the determination of catecholamines and their metabolites in rat brain extracts using fluorescent detection and derivatization with benzylamine and 1,2-diphenylethylenediamine [10]. Ragab et al. use 1,2-bis(3-chlorophenyl)-ethylenediamine to obtain chemiluminescent molecules [11]. The oxidation properties of the catecholamines can be used for their detection. Numerous articles describe the determination of catecholamines with electrochemical (EC) detection [5-7, 14, 15]. The limits of detection for these methods vary, but high sensitivity is achieved in several methods. Best sensitivity in electrochemical detection requires optimal operation conditions of the electrochemical detector, and the mobile phase has to be conductive, restricting the choice of its composition.

MS has become more and more popular as detection method [3, 4, 8, 16]. It has the advantage of being specific and offers the possibility of structural determination. On the other hand, MS methods suffer of poorer sensitivity than obtained with some methods using EC.

1.3 Micro – liquid chromatography

Microcolumn LC (μLC) was introduced by Horváth et al. in 1967 [17]. A review on μLC is given by Vissers [18], who describes theoretical and historical aspects as well as recent developments in μLC both concerning column and instrumental development. A recent article dealing with capillary columns was written by Takeuchi [19]. Both articles describe advantages of μLC over conventional LC. The smaller inner diameter of capillary columns (e.g. 300 μm) results in increased mass sensitivity. Because of the narrow diameter lower flow rates are used, making coupling to MS-detection without flow splitting easier and, because of lower consumption of mobile phase, exotic eluents can be used. The decrease in column diameter also gives rapid response to changes in temperature. Dilution on the column is reduced and thus the analyte concentration in the peak increases resulting in increased sensitivity. Smaller injection volumes are used, making it attractive for samples with limited access. Care has to be taken to minimize all connections and volumes in the injector and detector.

1.4 Large volume injections

One problem with μLC is the loss in detection sensitivity due to small injection volumes which is required to get high efficiency. However in μLC, larger volumes can be injected using column-switching methods based on on-column focusing on a precolumn and still maintaining the chromatographic efficiency. On-column focusing occurs when the solutes are dissolved in a non-eluting solvent [20, 21]. The solutes are concentrated in a narrow band at the entrance of a precolumn and are then backflushed onto an analytical column. The retention on the analytical column should be relatively higher than on the precolumn to obtain refocusing of the analytes at the column inlet. The concentration step on the precolumn can simultaneously provide sample purification since compounds that are not retained on the column are eluted into waste. Thus, such a column-switching method is desirable for complex brain samples.

1.5 Hypercarb stationary phase

Porous graphitic carbon was introduced as stationary phase (SP) for LC by Knox et al. in 1982 [22]. The stationary phase is now available under the trade name Hypercarb.

Hypercarb has great advantages over silica based stationary phases [23]: no swelling, shrinking or dissolution is observed with the use of aggressive eluents or 100% water mobile phases; it is stable in the entire pH-range; extreme conditions such as high salt concentrations or high temperature can be used; it has normal phase as well as reversed-phase properties.

Porous graphitic carbon stationary phases offer different retention mechanisms than C_{18} stationary phases [24]. The compounds are retained by complimentary mechanisms, e.g. hydrophobic and electronic interactions. Charge induced interactions e.g. electron-pair donor-acceptor and dipole-dipole induced types of interaction of the polarizable or polarized functional groups of the analyte with the polarizable stationary phase are believed to be the most important electronic interactions. The delocalization of the π-electrons in the graphite and the high polarizability of the carbon are responsible for dispersive interactions between the analyte and the stationary phase [24]. When stereochemistry forces the polar groups of the analytes to be close to the graphite surface, dispersion is particularly strong and the retention is influenced. Porous graphitic carbon consists of sheets of hexagonally arranged carbon atoms with sp^2 hybridisation, which results in strong π-π interactions with analytes containing aromatic rings. Thus, analytes containing aromatic rings are better retained than on a C_{18}-

material. Hennion et al. [24] concluded that for the retention of benzene rings with polar substituents electronic interactions are more important than hydrophobic interactions.

These retention mechanisms make Hypercarb attractive for the separation of polar compounds. A review of the separation of polar compounds on carbon stationary phase is given by Hanai [25]. Separation of inorganic ions [26-28] and positional and optical isomers [29, 30] have also been demonstrated.

1.6 Electrospray – time-of-flight – mass spectrometry

Mass spectrometric methods are specific, which makes them attractive in methods for analysis of samples containing a great variety of compounds, where not all of the analytes can be separated. Mass spectrometry provides an extra separation dimension to the method. Additionally, MS can provide structural information.

Electrospray ionization is a soft ionization technique. It is mainly used for charged analytes in a solution (e.g. the mobile phase). The main masses observed in an ESI mass spectrum using positive mode are the protonated molecular masses $[M+H]^+$. For higher molecular weight compounds, multiply charged masses are observed ($[M+nH]^{n+}$) [31]. Fragments and cluster ions (Na, K, formate, acetate) occur as well.

The time-of-flight mass analyzer detects all ions simultaneously. Extracted ion chromatograms (EICs) can be obtained form the total ion current (TIC). The time-of-flight mass analyzer achieves high speed; it has a good precision, high resolution and is suitable for molecules with high molecular weight [32].

1.7 Aim of study

Brain samples are complex and the concentration of catecholamines is low compared to other compounds. A concentration of catecholamines in rat brain tissue of up to 50-100 pg/ 20 mg brain has been reported [33]. Thus a method that is both sensitive and can separate a complex sample has to be used for their determination. A µLC method with large volume injection coupled to MS detection would be a suitable method.

The aim of this study was to develop a µLC column-switching method for the determination of low concentrations of dopamine, epinephrine, norepinephrine and serotonin in rat brain samples with ESI-MS as detection method.

In a two-dimensional method developed by Storbråten [34] for the determination of neuropeptides in rat brain samples, compounds with little retention on the reversed-phase precolumns of the second dimension were eluted to waste. These waste-fractions containing a lot of small, polar compounds have to be analyzed with a separate method. Originally, the goal was to develop a method for the determination of the compounds in these fractions. Since not all of the analytes of interest could be concentrated on the precolumn, it was decided to focus on catecholamines only. Unfortunately, problems with oxidation of both the stationary phase as well as the analytes halted the development of the method. Efforts were focused upon suppressing or preventing of oxidation to get a repeatable method. Therefore, analysis of real samples is not included in this study. The problems and limitations with the use of Hypercarb stationary phase are addressed.

2. Experimental

2.1 Reagents

Dopamine (D, 3-hydroxytyramine hydrochloride), epinephrine (E, (-)-epinephrine), norepinephrine (NE, (-)-arterenol, ≥98%) and serotonin (SE, 5-hydroxytryptamine maleate salt) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Grade 1 water was obtained from a Milli-Q ultrapure water purification system (Millipore, Bedford, MA, USA). For validation of the method, water was purchased from Fluka (Buchs, Switzerland).

HPLC grade acetonitrile (AcN) from Rathburn (Walkerburn, UK), sds (Peypin, France) or LabScan (Dublin, Ireland) was used. Methanol (MeOH, HPLC grade) was obtained from BDH Hipersolv (Poole, England). Tetrahydrofuran (THF, HPLC grade) was purchased from sds.

Acetic acid (AcOH, 100% analytical grade), trifluoroacetic acid (TFA, spectroscopic quality), ascorbic acid (≥ 99.7%, analytical grade), sodium thiosulphate (99.5-100.5%, analytical grade), hydrochloric acid (37% fuming, analytical grade) and benzoic acid (99.9%, analytical grade) were obtained from Merck (Darmstadt, Germany).

Pentafluoropropionic acid (PFPA, ≥ 97%), formic acid (FA, 50%, analytical grade) and ammonium formate (≥ 99.0%, puriss analytical grade for MS) were purchased from Fluka.

Heptafluorbutyric acid (HFBA, ≥98%) was obtained from Sigma-Aldrich.

Nitrogen (2.0, 99.99%) and helium (4.6, 99.996%) were purchased from AGA (Oslo, Norway).

2.2 Instrumentation

2.2.1 Column – switching system

A sketch of the µLC system is shown in figure 2.

All capillaries were either fused silika capillaries purchased from Polymicro Technologies (Phoenix, AZ, USA) or PEEK tubing (Upchurch scientific, Oak Harbor, WA, USA). Various dimensions and lengths were used throughout the system, they are shown in figure 2. Capillary connections were made with Valco Instruments Co. (Houston, TX, USA) ZU1C unions, Valco FS1.4 polyimide ferrules and Valco 2SR1 steel screens. The injector was a Rheodyne (Cotati, CA, USA) 7010 6 ports injector (valve I). External sample loops with volumes of 25, 50, 100 and 200 µl were prepared from PEEK tubing. A 250 µl laboratory syringe from SGE (Ringwood, Victoria, Australia) was used as injection syringe. A ChemInert 2-position 6-port micro-valve (C2-1036) from Valco served as a column-switching valve (valve II). The loading mobile phase was delivered at a flow rate of 25 µl/min by a LC-10AD VP double head isocratic pump from Shimadzu Corporation (Kyoto, Japan). An Agilent 1100 capillary gradient pump (Palo Alto, CA, USA) with an incorporated on-line vacuum degasser was used to deliver the separation mobile phase. The flow rate of the separation mobile phase was 5 µl/min. The outlet of the analytical column was connected via an union which served as grounding point to a Micromass (Manchester, UK) LCT orthogonal accelerated TOF-MS. The TOF-MS was equipped with a Z-spray atmospheric pressure ionization source for ESI, which was modified with a spray capillary of 25 µm i.d. to handle flow rates in the low µl/min range. Electrospray ionization was performed in the positive mode and the applied voltages are shown in table 1. A more detailed description of the MS-conditions is given in table A4 in the appendix.

Table 1: Applied MS–voltages.

Part	*Voltage [V]*
Spray capillary	2800
Sample cone	15
Extraction cone	3
RF lens	200

The source temperature was 100°C. The desolvation gas flow rate was ~250 l/h. The mass-to-charge-ratio (*m/z*) range during acquisition was set to 135 – 195. The TOF-MS instrument was controlled and data were acquired using MassLynx version 4.0 software (Micromass).

In-line UV detection was performed with a WellChrom K-2600 UV detector (Knauer, Berlin, Germany) equipped with a SunChrom (Friedrichsdorf, Germany) 10 mm, 0.045 µl SC-Cap-flow cell.

All voltametric measurements were made with a Fluke (Everett, WA, USA) 73 Series II multimeter.

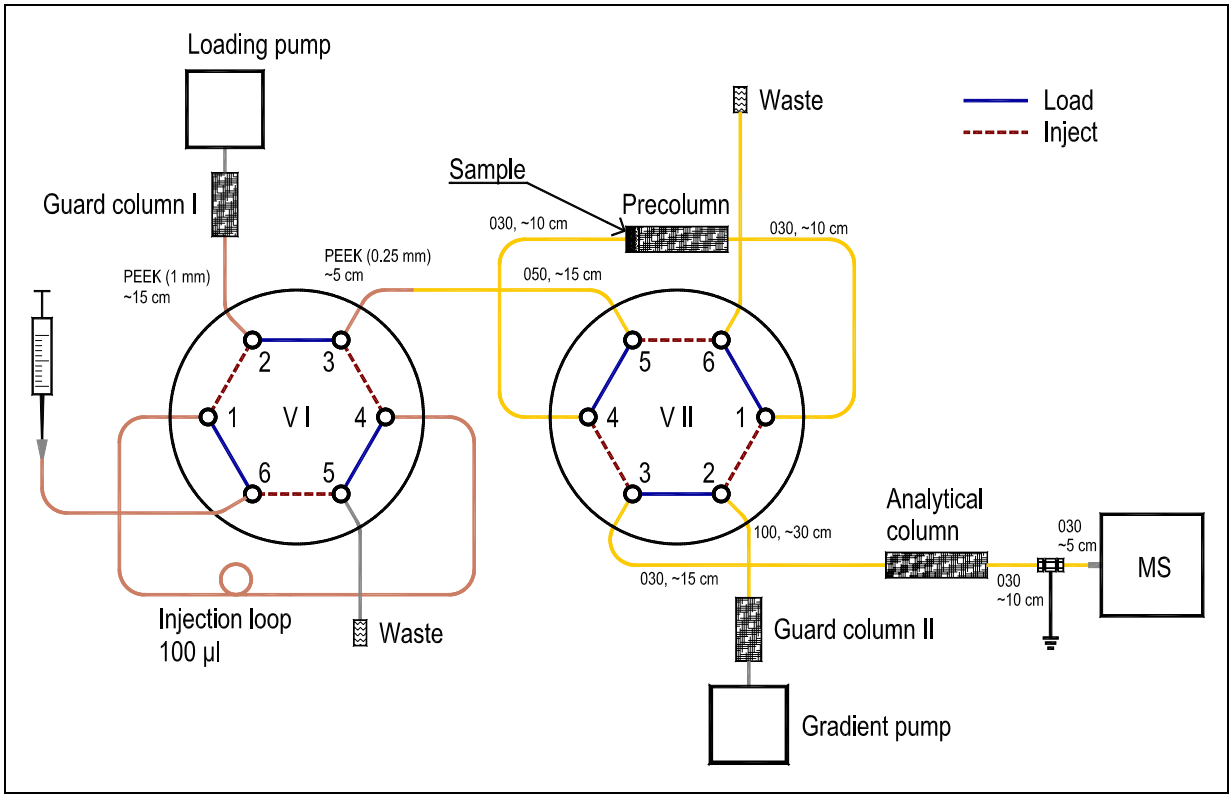


Figure 2: Sketch of the column-switching system. Yellow: Fused silika capillaries; brown: PEEK tubing; grey: steel tubing; 030 = 30 µm i.d.; 050 = 50 µm i.d.; 100 = 100 µm i.d.

2.2.2 Simplified instrumentation

During parts of the method development a simplified system was used. A sketch of the system is shown in figure 3. A Valco ChemInert C4-0004-.05 injection valve with an internal 50 nl loop was used as injection valve. The gradient pump delivered mobile phase with a flow rate of 5 µl/min. Only the analytical column was used. Detection was performed by (+) ESI-MS.

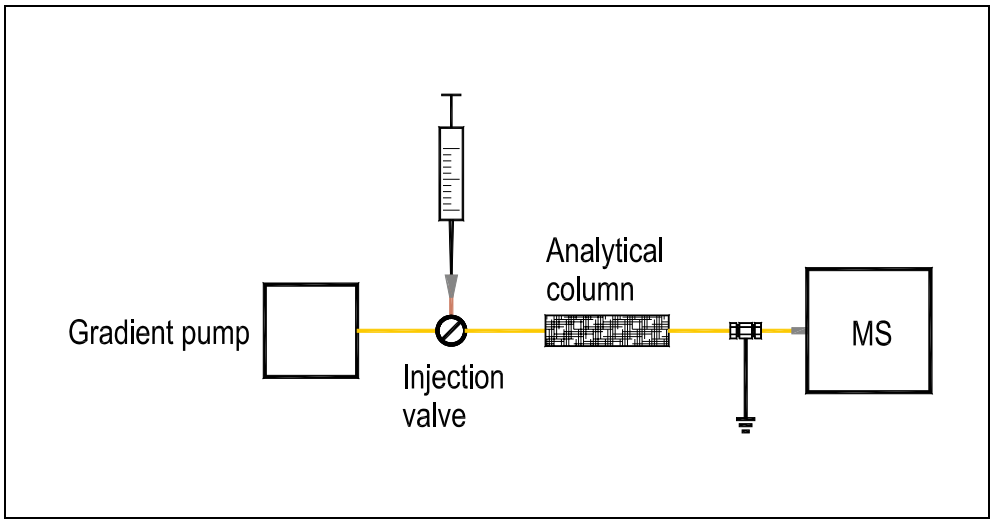


Figure 3: Sketch of the simplified system.

2.3 Columns and column packing

Dimensions of the columns used in the system are shown in table 2. In addition, three columns tested as possible precolumns are included (marked *).

Table 2: Dimensions of the Hypercarb columns used in the final system and column dimensions of three different precolumns.

Column type	*i.d. [mm]*	*o.d. [mm]*	*Length [cm]*	*Pore size [Å]*	*Particle size [µm]*
Precolumn	0.32	0.45	2.5	250	5
Analytical column	0.32	0.45	3.4	250	5
Guard column I	0.32	0.45	~ 3	250	5
Guard column II	0.32	0.45	~ 2.5	250	5
**Kromasil C_{18}*	0.32	0.45	4	100	10
**Hypercarb*	0.32	0.45	4	250	5
**PS-DVB*	0.2	not known	5	monolithic	monolithic

Hypercarb batch PGC 221, 5 µm (Thermohypersil, Chesire, UK) was used as stationary phase material in all Hypercarb columns. The Kromasil material was obtained from Eka Nobel (Bohus, Sweden). The polystyrene-divinylbenzene column was purchased from LC Packings (Amsterdam, The Netherlands). The columns were slurry packed using a mixture of AcN and grade 1 water (70/30, v/v) as packing fluid. About 30 mg packing material was suspended in 200 µl carbon tetrachloride and kept in an ultrasonic bath for 10 min to get a proper suspension. The suspension was introduced into a metal tubing (packing chamber) connected via a Valco ZU1C union to a fused silica capillary with a length of about 5-7 cm. The other end of the capillary was again connected to a union with a Valco 2SR1 frit to keep the packing material inside the capillary. The top of the packing chamber was connected to an ISCO 100DM syringe pump. The pump was programmed to start at 100 bar and increase the pressure at a rate of 200 bar/min to 650 bar. At 650 bar, the column was conditioned for 20 min. After the conditioning step the pump was disconnected with a valve. The column was allowed to depressurize for another 20 min. The pressure in the system was decreased by another program starting at 650 bar with a rate of -100 bar/min to 1 bar. After this the column was disconnected and cut into appropriate lengths.

2.4 Preparation of solutions

All solutions containing catecholamines were prepared in Plastibrand PP 1.5 ml plastic vials (Brand GMBH, Wertheim, Germany) or in 20 ml PE vials (Zinsser, Frankfurt, Germany). Microliter pipets were always used to deliver microliter volumes.

2.4.1 Mobile phases

Mobile phases for the loading of samples were prepared using a measuring cylinder; mobile phases for the analytical separation were prepared using glass volumetric flasks. Table 3 shows the mobile phases used in the final method and during testing of the precolumns. In the final method, the mobile phases were de-gassed with helium for ~15 min prior to use.

Table 3: Mobile phases.

Type	*Mobile phase*	*Type*	*Mobile phase*
loading	99.9% H_2O + 0.1% PFPA	*test precolumn (A1)*	99.9% H_2O + 0.1% AcOH
separation (A)	99.9% H_2O + 0.1% AcOH	*test precolumn (B1)*	99.9% AcN + 0.1% AcOH
separation (B)	99.9% AcN + 0.1% AcOH	*test precolumn (A2)*	99.9% H_2O + 0.1% HFBA
		test precolumn (B2)	50% AcN + 0.1% HFBA

2.4.2 Stock solutions

1 mg/ml stock solutions of each of the four catecholamines were prepared by dissolving 1 mg of substance in 1 ml 50:50 H_2O:0.1 M HCl (v/v). The stock solutions were stored in darkness at -20°C and had a shelf life of at least a month [4].

2.4.3 Direct infusion solutions

Direct infusion solutions of each catecholamine (table 4) for the determination of exact molecular masses and the optimization of the ESI-MS conditions were prepared. Each solution contained 0.1 mg/ml analyte, an appropriate amount AcN and 0.1% acetic acid to get ionization of the test substance and to control the pH. To compare signal-suppressing effects of four different ion-pairing agents, four direct infusion solutions containing 0.1% of a respective ion-pairing agent, 0.1 mg/ml of each catecholamine and 99.9% water were prepared. The direct infusion solutions were stored in darkness at 4°C.

Table 4: Direct infusion solutions for optimization of MS conditions.

	Dopamine	*Epinephrine*	*Norepinephrine*	*Serotonin*
Concentration [mg/ml]	0.1	0.1	0.1	0.1
% AcN	10	10	10	50
% AcOH	0.1	0.1	0.1	0.1

2.4.4 Working solutions and validation solutions

Working solutions for method development were prepared by appropriate dilutions of the stock solutions with grade 1 water. Validation solutions were prepared by appropriate dilutions of the stock solutions with water from Fluka. Table 5 shows the concentration of working solutions and validation solutions. The solutions were stored in darkness at 4°C and could be stored for at least a week [2].

The linearity test was performed by injecting validation solutions from lowest to highest concentration to avoid carry-over: blank, validation solution #1 (n = 3), validation solution #2 (n = 1), validation solution #3 (n = 3), validation solution #4 (n = 1), validation solution #5 (n = 3), validation solution #6 (n = 1). The within-day and between-day precisions were calculated by 3 series of validation solutions at three concentration levels (1, 3 and 5, n = 3) on 3 different days. A blank was included at the beginning of each series to recondition the system. The guard columns were treated with 100 µl 1 M $Na_2S_2O_3$ and 150 µl water after each day to regain their original properties.

Table 5: Concentration of working solutions and validation solutions.

Solution	*Concentration [ng/ml]*			
	Dopamine	*Epinephrine*	*Norepinephrine*	*Serotonin*
Working solution 50 nl	10^4	10^4	10^4	10^4
Working solution loop	250	250	250	250
Working solution loop	62.5	62.5	62.5	62.5
Blank	0	0	0	0
Validation solution #1	7.5	3.0	10	10
Validation solution #2	15	6.0	20	20
Validation solution #3	25	15	30	30
Validation solution #4	35	30	50	50
Validation solution #5	50	50	60	60
Validation solution #6	70	70	125	125

2.4.5 Other solutions

A 1 M ammonium formate solution was prepared from solid formic acid ammonium salt. The pH was adjusted to 2.9 with formic acid. A 1 M sodium thiosulphate solution was prepared from solid sodium thiosulphate (NaS_2O_3 $5H_2O$). A 1 M ascorbic acid solution was prepared from solid L-(+) ascorbic acid and 5 mM benzoic acid was prepared from solid benzoic acid. All solutions prepared form salts were filtered through a 0.45 µm filter (Sartorius AG, Göttingen, Germany) before use.

3. Results and discussion

The concentration of catecholamines in brain samples is low compared to other compounds. Additionally, brain samples have a complex matrix with a great variety of compounds. Thus a selective method with a low limit of detection capable to give separation of a complex sample has to be used for the determination of catecholamines in brain samples. Sensitivity is achieved by using a µLC column-switching method and ESI-MS which provides both selective and sensitive detection and is therefore chosen as detection method.

3.1 Mass spectrometric detection

The masses used for detection were determined by direct infusion. Figure 4 shows the mass spectra for dopamine (a), epinephrine (b), norepinephrine (c) and serotonin (d). The main *m/z* peaks are the protonated molecular ions, $[M+H]^+$. Fragments of mass $[M+H-17]^+$ due to loss of ammonia (- NH_3) for D and SE and fragments of mass $[M+H-18]^+$ due to loss of water (- H_2O) for E and NE were observed as well. The *m/z* values of the molecular ions and the fragments are summarized in table 6.

Both the molecular ion and the fragment ion are included in the extracted ion chromatograms (EICs) and the calculations of peak areas, if not otherwise is stated.

Table 6: *m/z* values of $[M+H]^+$ and *m/z* values of fragments determined by direct infusion (table 4).

	Dopamine	*Epinephrine*	*Norepinephrine*	*Serotonin*
m/z $[M+H]^+$	154.1	184.1	170.1	177.1
m/z fragment	137.1	166.1	152.1	160.1

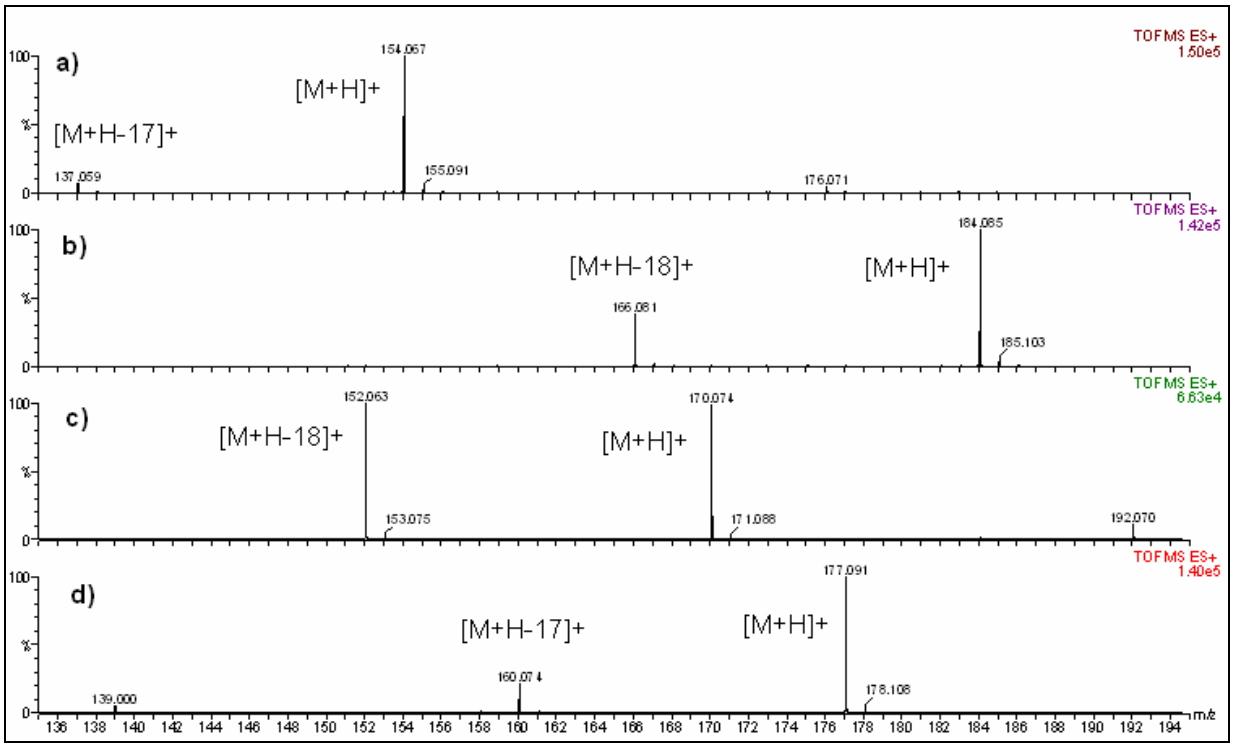


Figure 4: Mass spectra of direct infusion of catecholamines. **a)** D, 0.1 mg/ml, 10% AcN, 0.1% AcOH; **b)** E, 0.1 mg/ml, 10% AcN, 0.1% AcOH; **c)** NE, 0.1 mg/ml, 10% AcN, 0.1% AcOH; **d)** SE, 0.1 mg/ml, 50% AcN, 0.1% AcOH.

3.2 Stationary phase considerations

Care has to be taken when ESI-MS detection is used in the work with Hypercarb columns. Electrochemical reactions at the grounding point of the backward current from the ESI-spray capillary enhance oxidation of the mobile phase, the analytes and the stationary phase material. To prevent oxidation of Hypercarb and the analytes by the backward current, a grounded stainless steel union has to be coupled between the ESI-spray capillary and the analytical column [35]. However, the stationary phase can also slowly be oxidized by some oxidizing agents or dissolved oxygen in the mobile phase [36], and care has to be taken to de-gas all mobile phases.

Oxidized Hypercarb material has different chromatographic behavior from reduced Hypercarb material, as demonstrated by Törnkvist and co-workers [36]. When Hypercarb is treated with oxidizing agents, such as permanganate or hydrogen peroxide, the retention times of cationic analytes were decreased [37]. Peak splitting of the peaks of easy oxidized analytes on an oxidized column was observed as well [35].

The different behavior of oxidized Hypercarb columns is explained by an alternation of the surface properties. The anion-exchange capacity of the Hypercarb column increases with

increased concentration of oxidizing agents. Thus exclusion of cationic analytes is exerted by the material, resulting in a decrease in retention times. This suggests that Hypercarb may have positive surface charge, which can be altered by treatment with redox agents. Treatment of Hypercarb with oxidizing agents increases the surface positive charge, explaining shorter retention times of cationic analytes [37]. The charge that the particles have gained was however not distributed evenly throughout the column. Thus, it was assumed that the redox reactions occurring on the Hypercarb column were caused by some functional groups on the surface of the stationary phase material that were altered by redox reactions [36]. When the column is exposed to oxidizing agents, a slow change of the Hypercarb material due to oxidation of these functional groups occurs. The effect of oxidation of Hypercarb lasts longer than the effect of reducing agents [37].

3.2.1 Precolumn

In order to obtain sufficient focusing of the polar catecholamines, a stationary phase which provides high retention of the compounds has to be used. Three different materials were considered as stationary phase, a C_{18}-material (Kromasil), porous graphitic carbon (Hypercarb) and monolithic polystyrene-divinyl benzene (PS-DVB). The Kromasil C_{18}-material was chosen because it was shown to provide most retention of the five different C_{18}-materials tested in preliminary experiments (appendix, chapter 6.1.1).

In a column-switching system it is crucial that the analytes are fully retained on the precolumn for their complete preconcentration. Thus breakthrough of various analytes on the precolumns was tested with a mobile phase containing an ion-pairing agent. The procedure is illustrated in figure 5. A more detailed description of experimental parameters and a table of all compounds tested are given in table A3 in the appendix.

Table 7 shows the conditions under which the catecholamines could be focused. Hypercarb turned out to be the best suitable stationary phase for concentration of the catecholamines on the precolumn. However, even on the Hypercarb column, AcOH did not give enough retention of D, E and NE, thus a more hydrophobic ion-pairing agent, had to be used for concentrating on the precolumn.

Table 7: Conditions for concentration of catecholamines on precolumns tested.

		Dopamine	*Epinephrine*	*Norepinephrine*	*Serotonin*
C_{18}	*AcOH*	no	no	no	no
	HFBA	no	no	no	no
Hypercarb	*AcOH*	no	no	no	yes (0% AcN*)
	HFBA	yes (0% AcN*)	yes (0% AcN*)	yes (0% AcN*)	yes (0% AcN*)

* The numbers in brackets give the minimum percentage AcN that can be present in the MP to get focusing of all four analytes.

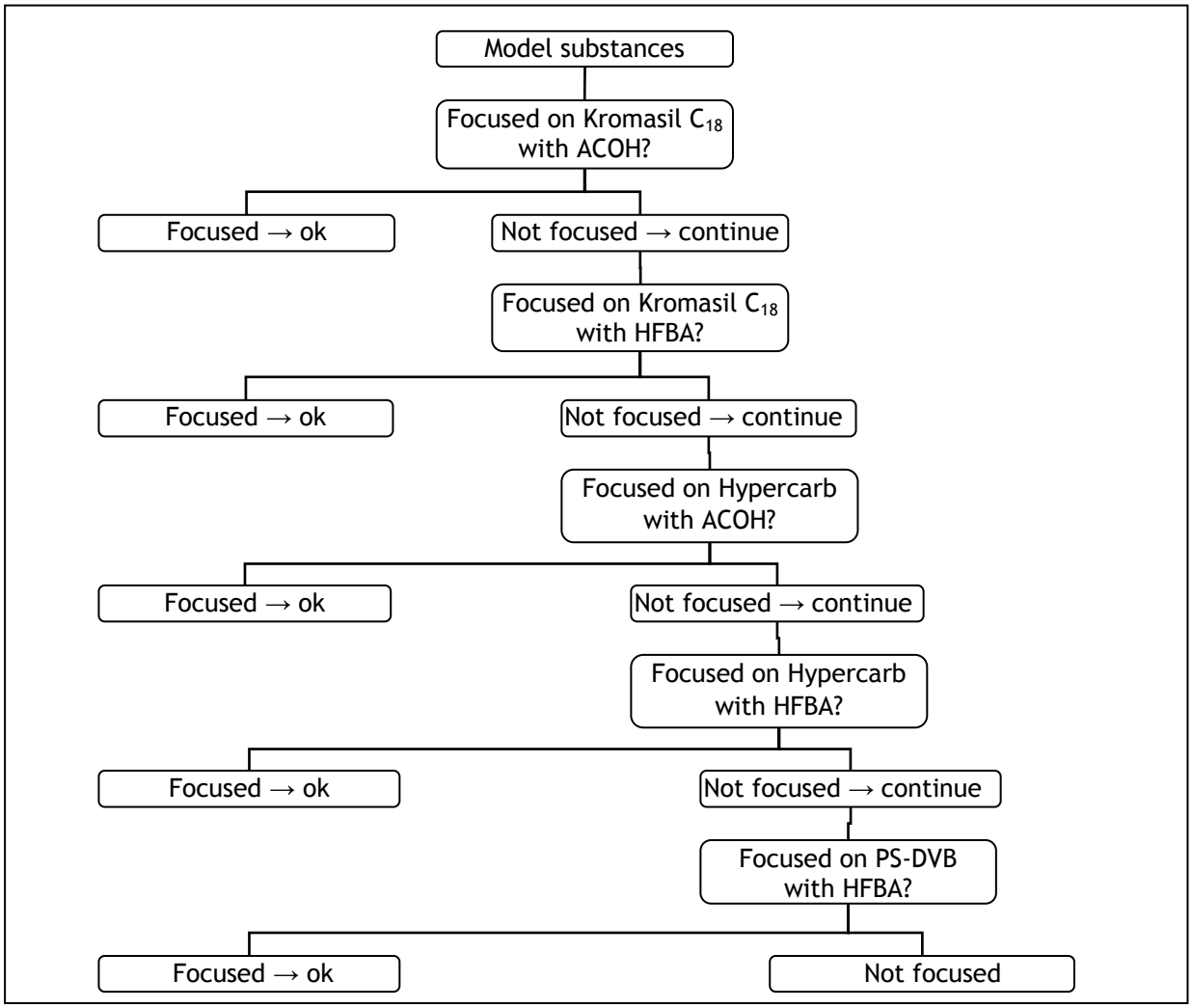


Figure 5: Flow chart illustrating the procedure for testing reversed-phase precolumn materials.

3.2.2 Analytical column

After backflushing from the precolumn it is advantageous to use a stationary phase in the analytical column which gives slightly higher relative retention than the precolumn. The Hypercarb material was only available in two particle sizes, 7 µm and 5 µm at the time. Thus, a particle size of 7 µm would have been desirable to use in the precolumn and to refocus the analytes on the analytical column (5 µm particles). However the 7 µm batch was severely contaminated with smaller, irregular sized particles, and could not be used as precolumn material [38]. Therefore, both the analytical column and the precolumn consisted of Hypercarb stationary phase material of the same particle size, 5 µm.

3.3 Mobile phase considerations

3.3.1 Separation mobile phase

The separation mobile phase was found using the simplified system (figure 3).

The most common organic modifiers used in liquid chromatography are methanol (MeOH), AcN and tetrahydrofuran (THF). MeOH and THF give higher backpressure due to higher viscosity in water mixtures than AcN. Since 3 columns are coupled in series during elution and separation in the final method, high backpressure is a problem with water/MeOH and water/THF mixtures. The highest pressure observed during analysis with a water/AcN mobile phase was ~250 bar. Thus MeOH and THF were not suitable in the final system.

An ion-pairing agent had to be added to the mobile phase to give high enough retention. Additionally, the mobile phase for ESI-detection of basic compounds should be acidic (two units below pK_a) to get ionization of the analytes and pH-control. AcOH, trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA) and HFBA were compared as possible ion-pairing agents. Direct infusion solutions were introduced into the MS and the extracted ion chromatogram yielded the signal intensities. The values of the signal intensities are shown in table 8.

Table 8: Signal intensities of *m/z*-values of the catecholamines with four different acids used as ion-pairing agents determined by direct infusion with a solution containing 0.1 mg/ml catecholamines. The intensities were obtained from EIC.

	Signal intensities [arbitrary units]			
Ion-pairing agent	*Dopamine*	*Epinephrine*	*Norepinephrine*	*Serotonin*
AcOH	22700	23500	13900	13800
TFA	907	999	707	540
PFPA	1260	1290	901	775
HFBA	2200	2810	1530	1190

Severe signal suppression was observed with TFA as ion-pairing agent. This has been described before [39], but the effect differs from instrument to instrument and analyte to analyte. PFPA gave severe signal suppression for NE and SE, while only moderate for D and E. HFBA gave moderate signal suppression for all four analytes. Formic acid (FA) could be an alternative, but better chromatographic peak shape is achieved with AcOH as ion-pairing agent.

Gradient elution was performed to separate the catecholamines on the analytical column. Gradient elution is in this case preferred over isocratic elution because of the great difference in retention times of the four catecholamines. D, E and NE have similar retention times, while SE has much higher retention. By applying a gradient, much better peak shape for SE was achieved and the chromatographic time was reduced.

The unique retention mechanisms of Hypercarb columns induce an elution of each catecholamine from the column at a specific amount AcN. The interaction strength of the catecholamines with the graphite is dependent of the molecular area in contact with the column surface. The planar structure of the catecholamines gives a strong interaction, which only can be broken with a specific amount AcN. Thus, sharp peaks are obtained as can be seen in figure 6.

In table 9 the best separation gradient is presented. NE, E and D are eluted in the linear gradient of the first 2.5 min. SE is eluted in the 50% B step.

Table 9: Best separation gradient. A = 99.9% H_2O+0.1% AcOH. B = 99.9% AcN+0.1% AcOH.

Time [min]	*% B*	
00.00	0	initial condition, start ramp
02.50	10	end ramp
02.51	50	elution SE
05.00	50	
05.01	100	column wash (precolumn and analytical column)
10.00	100	
10.01	0	reconditioning* (analytical column)
15.00	0	

* The precolumn is reconditioned with 0% B for 2 min before the reconditioning with the loading MP starts.

A chromatogram using this gradient is shown in figure 6. The elution order is NE; E; D; SE.

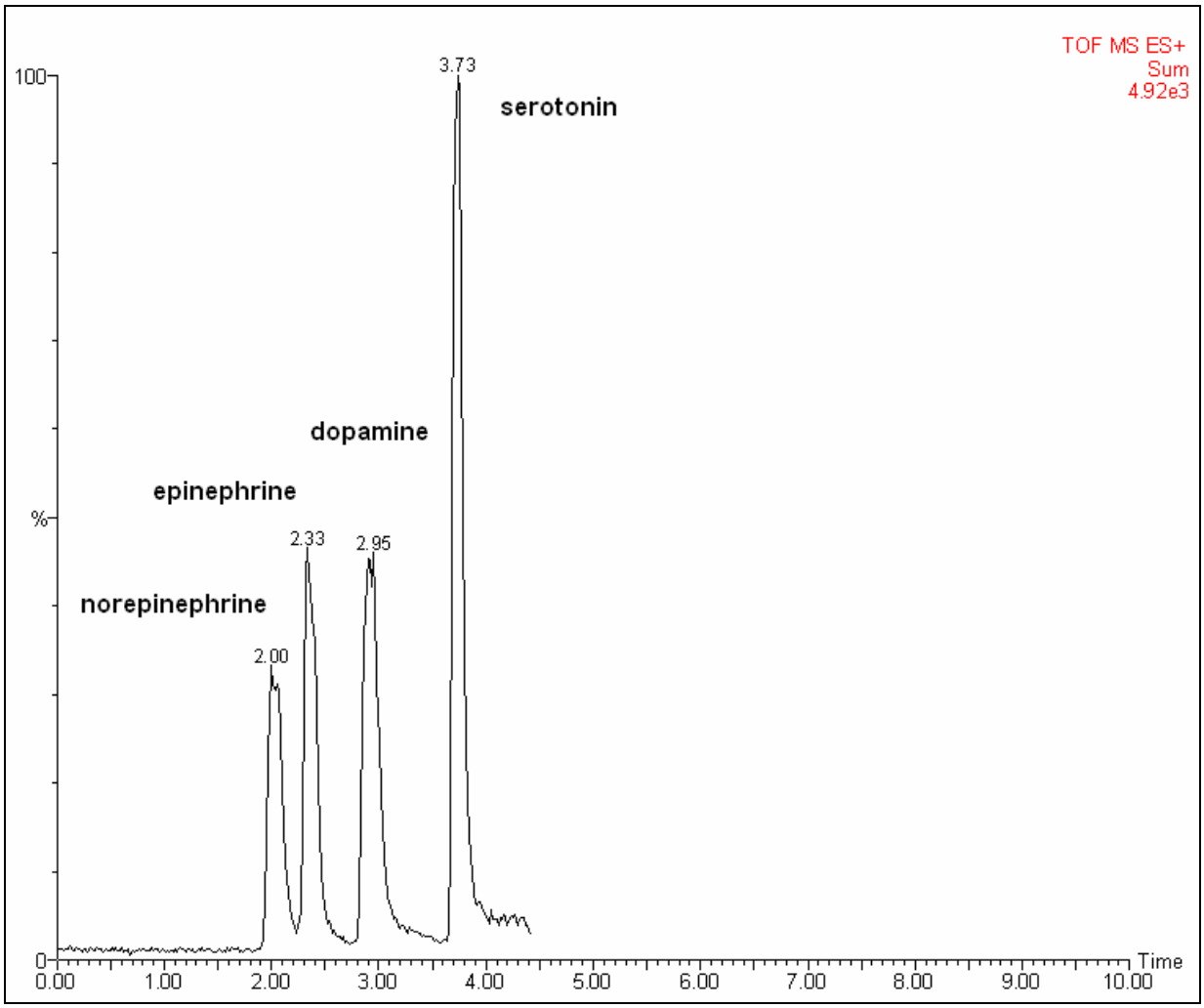


Figure 6: EIC of the separation of catecholamines using gradient elution on a 50 x 0.32 mm (i.d.) 5 µm Hypercarb column. The MP consisted of H_2O+0.1% AcOH (A) and AcN+0.1% AcOH (B) with a flow rate of 5 µl/min. The injection volume was 50 nl and the sample concentration 0.1 mg/ml of each catecholamine.

3.3.2 Loading mobile phase

To focus the catecholamines on the precolumn, a mobile phase consisting of 100% water added a suitable ion-pairing agent is needed, since small amounts AcN elute the analytes. Four acids, AcOH, TFA, PFPA and HFBA were tested as ion-pairing agents.

AcOH did not give enough retention of D, E and NE on the precolumn (chapter 3.2.1), thus a more hydrophobic ion-pairing agent had to be utilized. In the method developed by Storbråten [34], TFA is used to dilute the fractions from the first dimension. Thus, TFA in the loading mobile phase would have been ideal with regard to analyzing waste fractions originating from that method.

However, a low signal intensity was obtained for NE with 0.1% TFA in the loading mobile phase. By changing to 0.1% HFBA, the intensity of the NE signal was improved (figure 7). Thus it appears that TFA is not hydrophobic enough to provide satisfactory focusing of NE on the precolumn. The intensity of the SE signal was lower with HFBA than with TFA (not shown). This may be due to ion-pair binding of SE with HFBA which might be too strong and therefore the HFBA anion cannot be exchanged by AcOH used in the separation mobile phase. This might also be the reason for the somewhat higher retention observed for NE (figure 7). The amount HFBA was decreased to get a better exchange of the two ion-pairs, but no improvement was achieved. The use of 0.1% PFPA improved both the intensity of NE (figure 7) and SE, and thus PFPA was chosen as ion-pairing agent in the loading mobile phase.

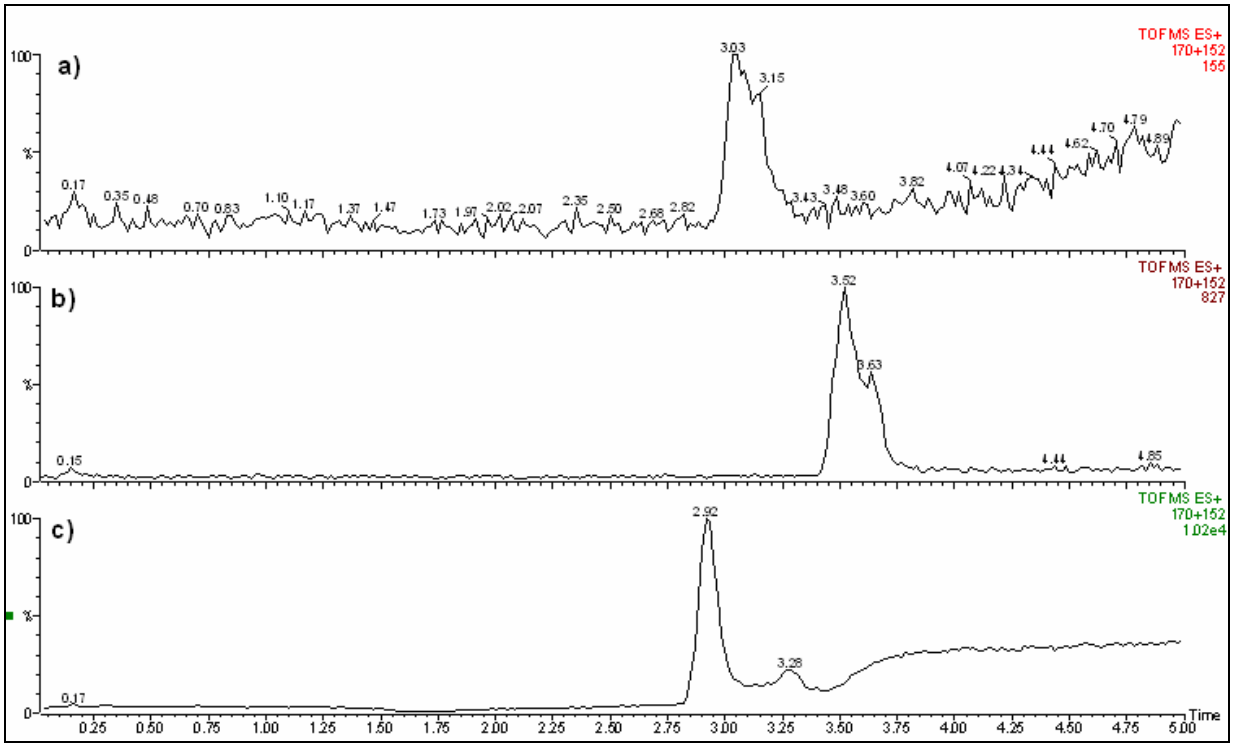


Figure 7: EIC showing the effect of the loading MP ion-pairing agent on signal intensity of norepinephrine. **a)** TFA; **b)** HFBA, **c)** PFPA.
The SP of both columns was Hypercarb. A sample concentration of 250 ng/ml was used and the sample loop had a volume of 25 µl. The loading MP had a flow rate of 20 µl/min. The separation MP consisted of H_2O/AcN added 0.1% AcOH with a flow rate of 5 µl/min.

3.4 Separation of catecholamines on Hypercarb material

During development of the separation gradient (chapter 3.3.1), numerous injections were performed on a freshly packed column using the simplified system and water/acetonitrile containing 0.1% AcOH as separation mobile phase. The mobile phase was not degassed at that time and the column was grounded as proposed by Törnkvist and co-workers [35] to avoid oxidation of the analytes. No oxidation of the analytes was observed even though large amounts mobile phase were pumped through the column.

However, when using the column-switching system, after some injections a low intensity was observed for the peaks of E and SE in the EIC (figure 8). The analytical column was tested with the simplified method, and the EIC revealed four peaks with high intensity corresponding to the catecholamines (figure 9a). Then the precolumn was tested in the same way, and only two peaks were observed in the EIC (figure 9b). The mass spectra of the peaks in figure 9b revealed most abundant masses of *m/z* = 170 (NE) and *m/z* = 154 (D).

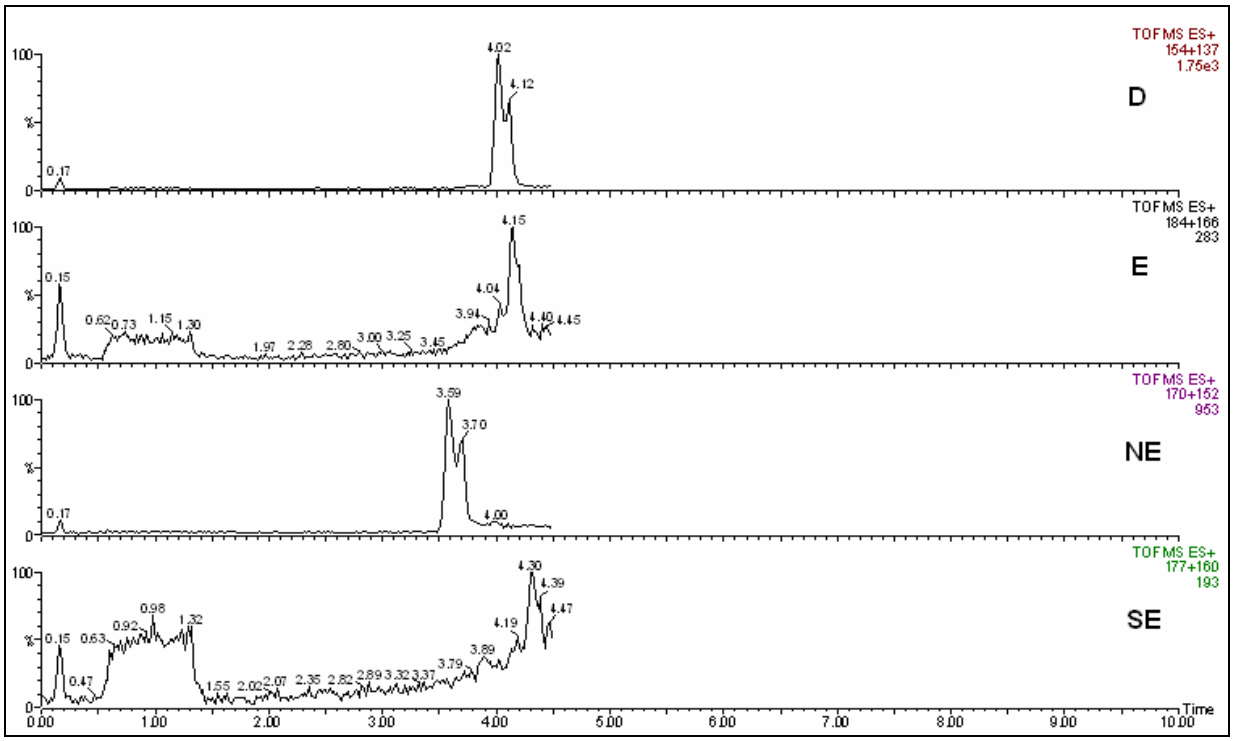


Figure 8: EIC of an injection in the column-switching system showing low signal intensity of the peaks of E and SE.
The SP of both columns was Hypercarb. A sample concentration of 250 ng/ml was used and the sample loop had a volume of 25 µl. The loading MP consisted of H_2O+0.1% PFPA with a flow rate of 20 µl/min. The separation MP consisted of H_2O/AcN added 0.1% AcOH with a flow rate of 5 µl/min.

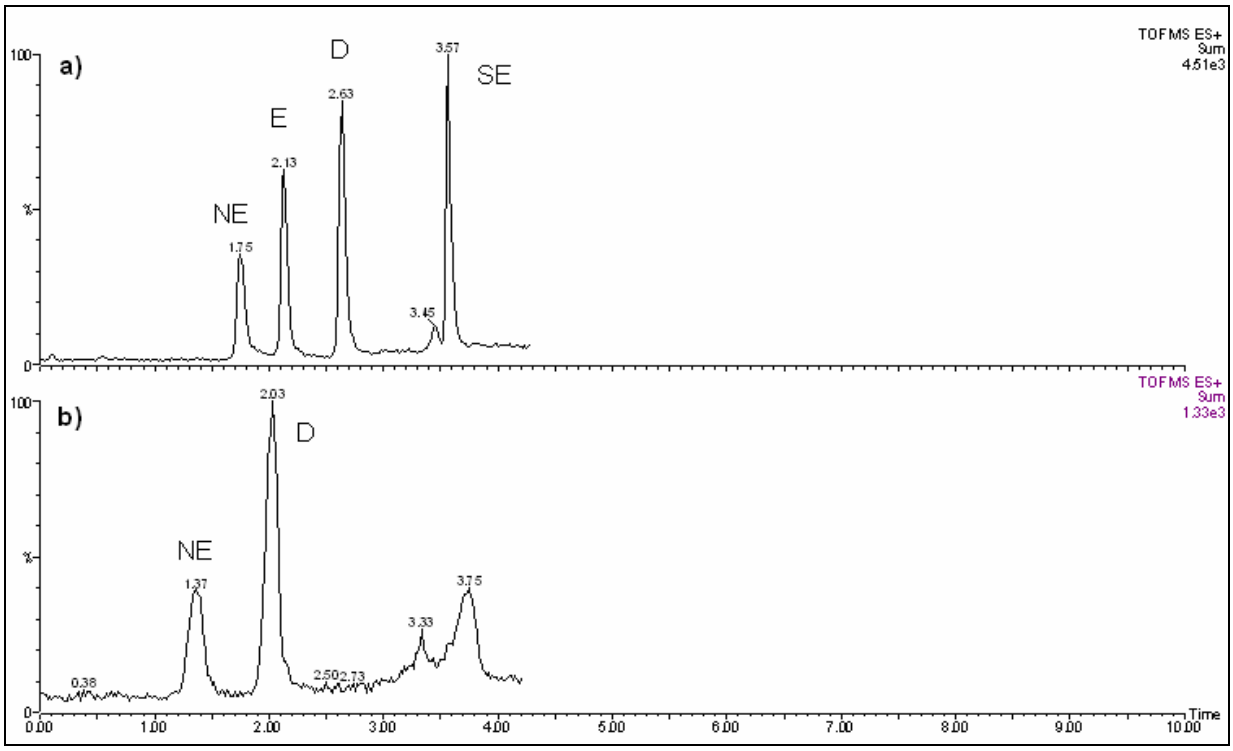


Figure 9: EIC of injections of catecholamines on the Hypercarb analytical column **(a)** and the Hypercarb trap column **(b)**.
The MP consisted of H_2O/AcN added 0.1% AcOH with a flow rate of 5 µl/min. The injection volume was 50 nl and the sample concentration 0.1 mg/ml of each catecholamine.

Obviously, some processes had occurred on the trap column in the column-switching system, since E and SE could not be detected with high intensity. Since the column was grounded it was not believed that oxidation had occurred. It was supposed that some irreversible processes involving the ion-pairing agent and the two analytes had occurred. An UV-detector was coupled in-line with the MS, to check if E and SE eluted from the precolumn. Four peaks were observed in the UV-chromatogram (Figure 10). Thus, some process on the column had altered the masses of at least E and SE.

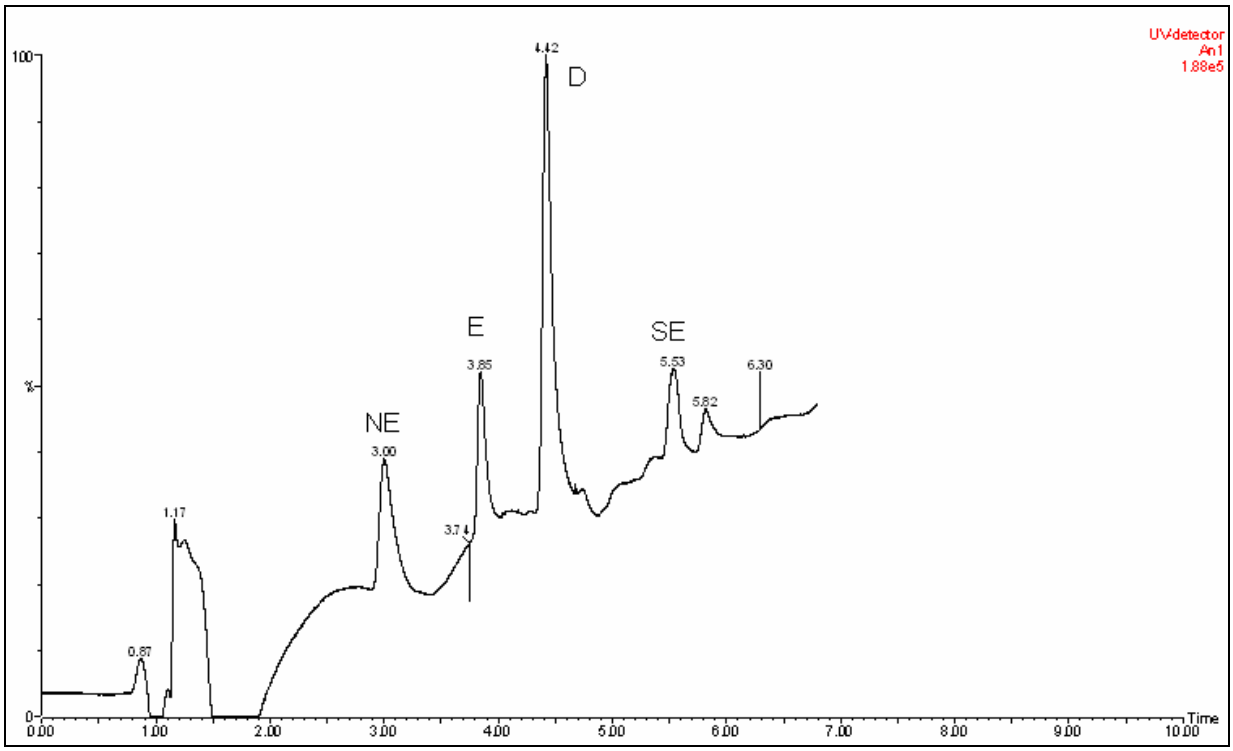


Figure 10: UV chromatogram of the column-switching method.
The SP of both columns was Hypercarb. A sample concentration of 250 ng/ml was used and the sample loop had a volume of 25 µl. The loading MP consisted of H_2O+0.1% PFPA with a flow rate of 20 µl/min. The separation MP consisted of H_2O/AcN added 0.1% AcOH with a flow rate of 5 µl/min.

Since no problems with detection were observed during the gradient tests (chapter 3.3.1), the retention times and signal intensities repeatability on the analytical column was tested with the simplified method in the same manner as during separation mobile phase evaluation. Figure 11 shows the chromatogram (TIC) for the first and fifth injection. After five injections, the E peak (2^{nd} peak) had nearly disappeared, while a peak with a retention time close to SE was observed (figure 11b).

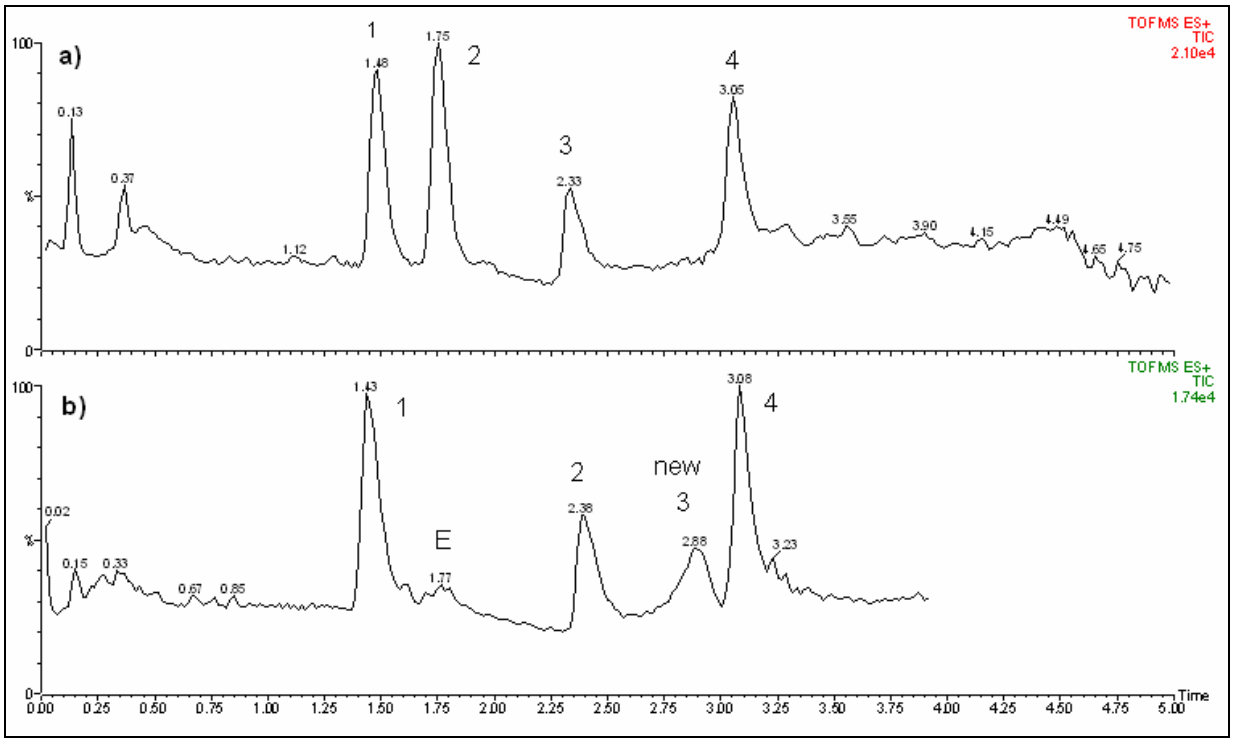


Figure 11: TIC of the first **(a)** and fifth **(b)** injection of a mixture of catecholamines on the analytical Hypercarb column alone. The MP consisted of H_2O/AcN added 0.1% AcOH with a flow rate of 5 µl/min. The injection volume was 50 nl and the sample concentration 0.1 mg/ml of each catecholamine.

Mass spectra of the compounds eluted in the four peaks observed in the TIC were obtained. They revealed the most abundant *m/z*-values of the peaks in the first injection were *m/z* = 170.1 (NE, 1st peak), *m/z* = 184.1 (E, 2nd peak), *m/z* = 154.1 (D, 3rd peak) and *m/z* = 191.1 (4th peak) (figure 12). The most abundant *m/z*-values of the peaks observed in the TIC of the 5th injection were *m/z* = 168.1 (1st peak), *m/z* = 152.1 (2nd peak), *m/z* = 180.1 (3rd peak) and *m/z* = 191.1 (4th peak) (figure 13).

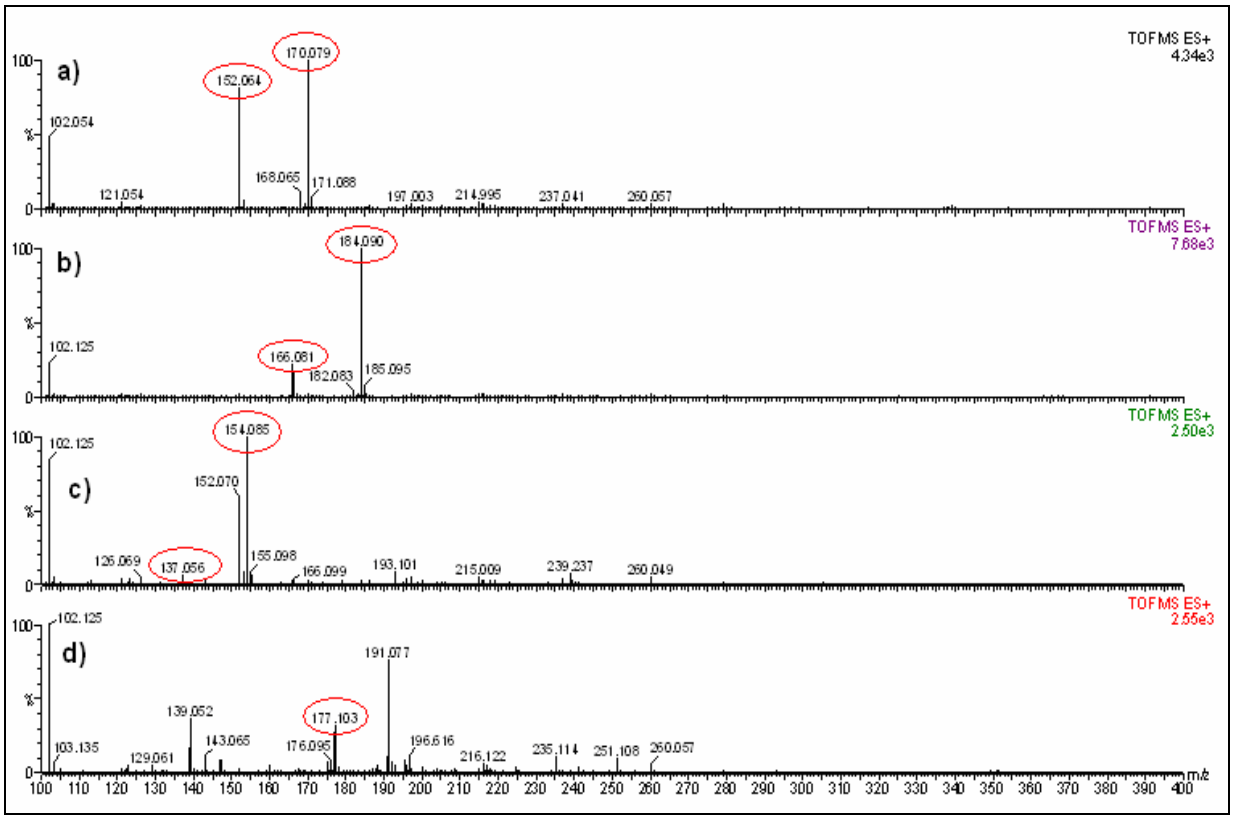


Figure 12: Mass spectra of the **a)** 1st peak, **b)** 2nd peak, **c)** 3rd peak, **d)** 4th peak observed in the TIC of the first repeatability test. *m/z* values of the catecholamines are marked with red.

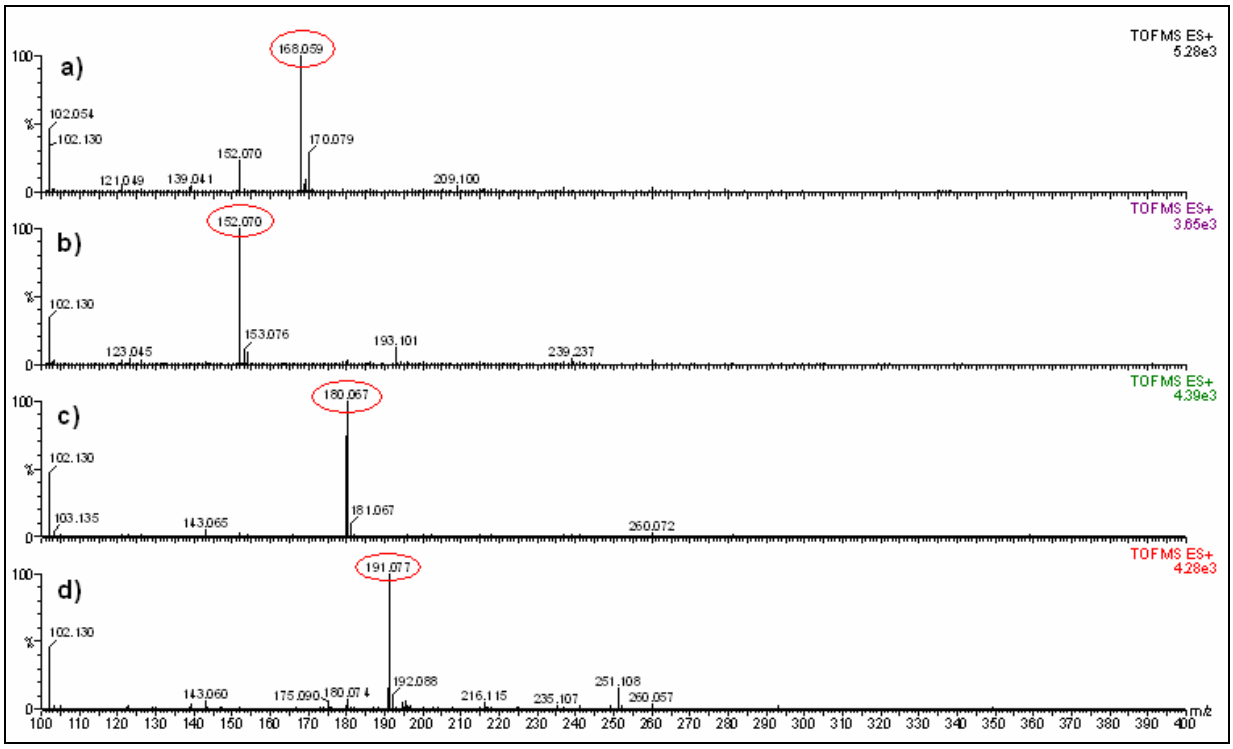


Figure 13: Mass spectra of the **a)** 1st peak, **b)** 2nd peak, **c)** 3rd peak, **d)** 4th peak observed in the TIC of the first repeatability test. The most abundant *m/z*-values are marked with red.

Injections of solutions containing only one of the catecholamines (figure 14) revealed that the 1st peak corresponded to NE ($[M+H-2]^+$), the 2nd to D ($[M+H-2]^+$), the 3rd to E ($[M+H-4]^+$) and the 4th to SE ($[M+H+14]^+$).

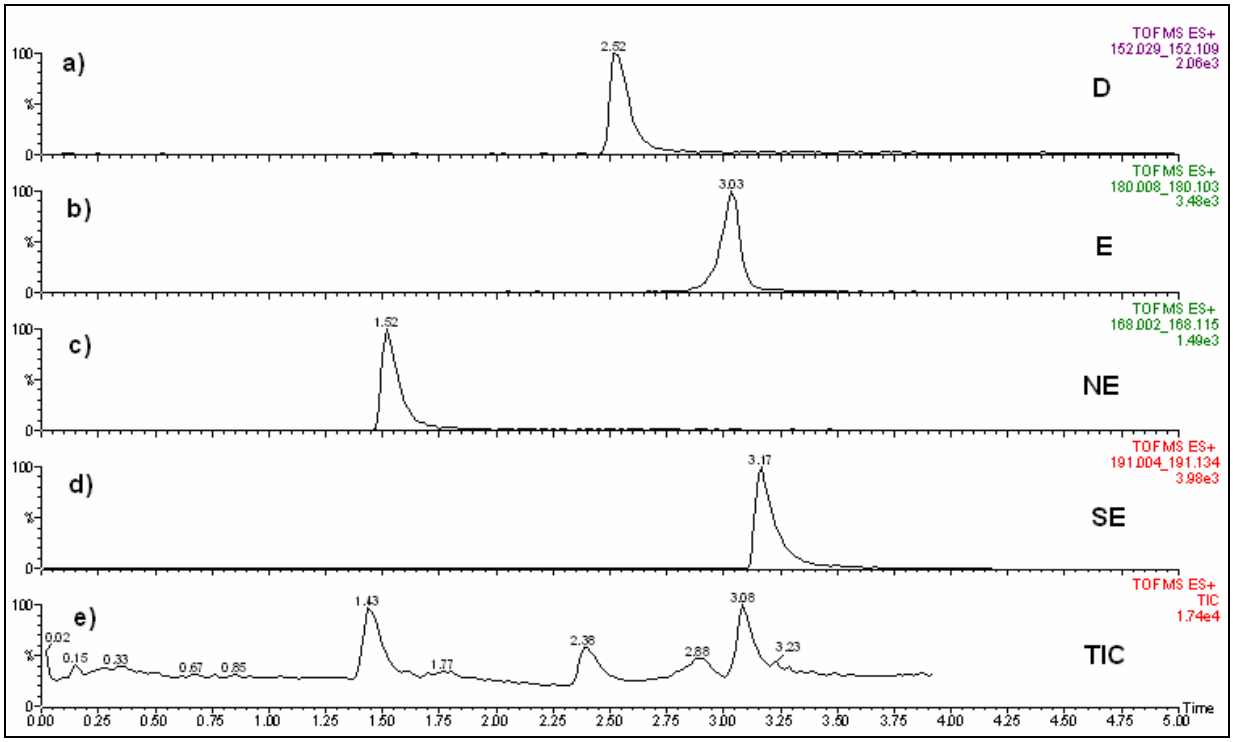


Figure 14: EIC **(a-d)** of injection of a solution containing D, E, NE or SE, respectively on the analytical Hypercarb column. TIC of the 5th injection on the analytical column **(e, figure 11b)**. The MP consisted of H_2O/AcN added 0.1% AcOH with a flow rate of 5 µl/min. The injection volume was 50 nl and the sample concentration 0.1 mg/ml of each catecholamine.

The *m/z*-values found suggested that oxidation of the catecholamines had occurred even though the column was grounded and no electrical current was measured over the column. The structures and masses of the oxidized catecholamines are given in figures 15-18. E is oxidized to adrenoquinone (*m/z* = 182) and leucoadrenoquinone (*m/z* = 180). Leucoadrenoquinone contains a double ring, thus an increase in retention time was observed (figure 11). The *m/z*–value of 191 for SE is believed to be indol-quinone as shown in figure 18 with an addition of one oxygen-atom to the molecule. A similar addition of oxygen has been observed for the amino acid tryptophane in water samples [40].

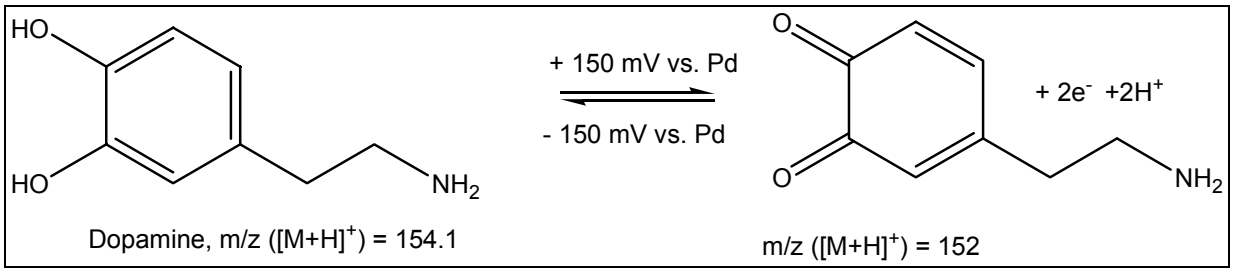


Figure 15: Oxidation of dopamine [41].
(ChemDraw Ultra 8.0 (CambridgeSoft Corporation, Cambridge, MA, USA))

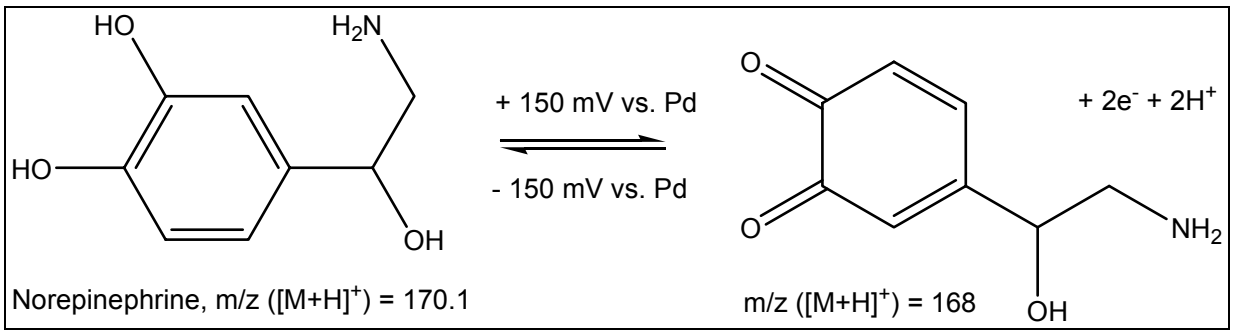


Figure 16: Oxidation of norepinephrine [41].
(ChemDraw Ultra 8.0 (CambridgeSoft Corporation, Cambridge, MA, USA))

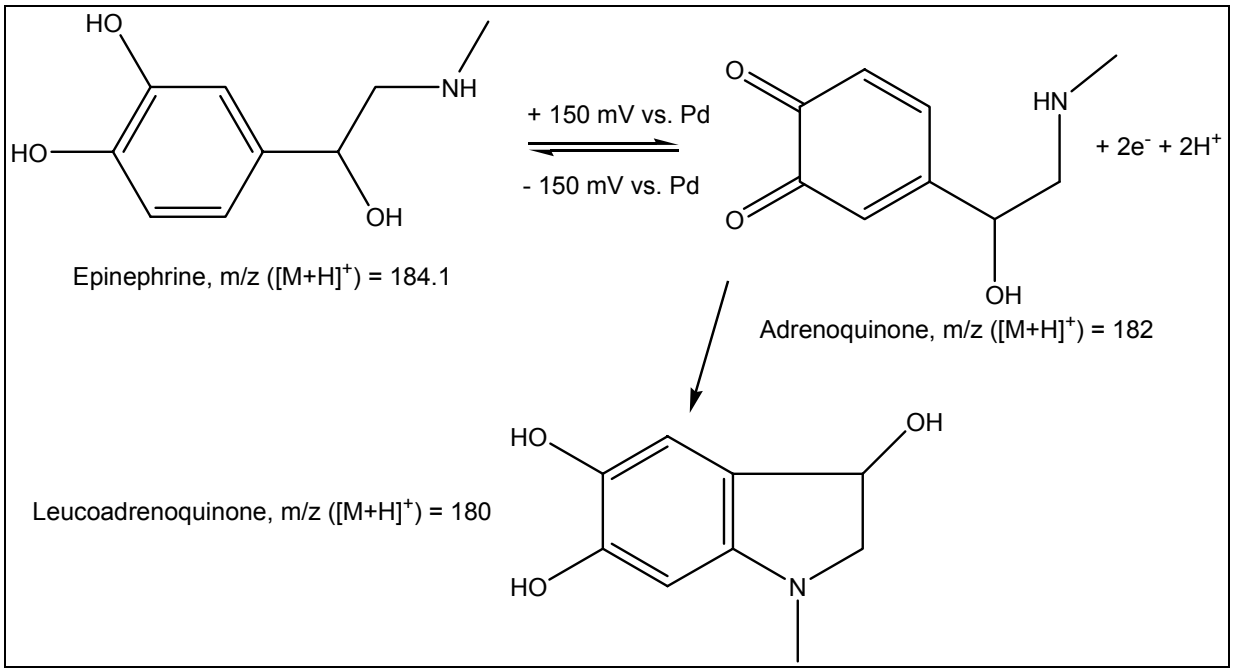


Figure 17: Oxidation of epinephrine [42].
(ChemDraw Ultra 8.0 (CambridgeSoft Corporation, Cambridge, MA, USA))

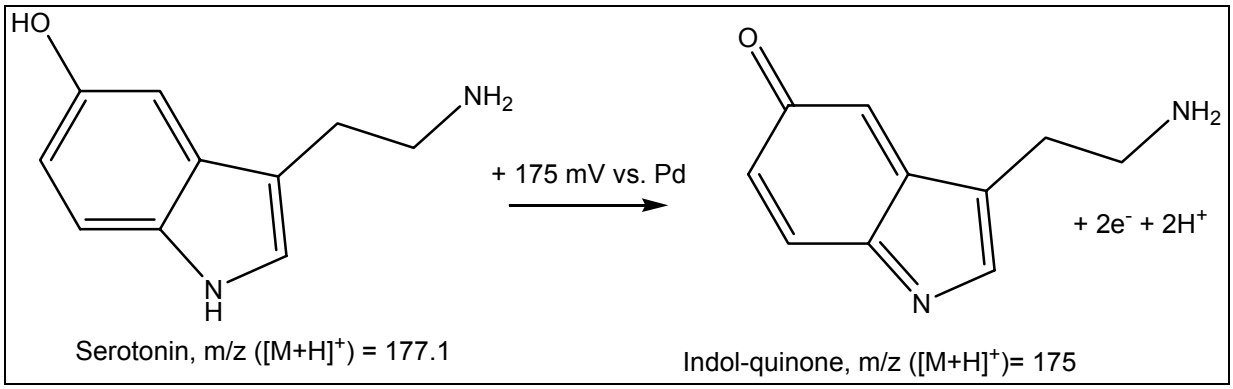


Figure 18: Oxidation of serotonin [41].
(ChemDraw Ultra 8.0 (CambridgeSoft Corporation, Cambridge, MA, USA))

As mentioned in chapter 3.2, it was believed, that oxidation of the Hypercarb material was caused by oxidation of functional groups on the surface of the stationary phase. The charge that the particles have gained during oxidation was not distributed evenly throughout the column. Since the oxidation of D and NE is reversible, they should be oxidized in one part and reduced in another part of the column.

However, it is shown that D was affected by the oxidized stationary phase. After 2-3 injections on the column, most D eluted as the quinone with molecular ion $[M+H-2]^+$ (figures 13 and 15) When the mobile phase used by Törnkvist and co-workers (MeOH:50 mM ammonium formate buffer (pH 2.9) 60:40 (v/v)) [16] was applied, oxidation of the catecholamines was observed as well. On the other hand, a newly packed Hypercarb column could be used with the separation mobile phase for several weeks without observing oxidation. However, once the columns were used in the column-switching system, the catecholamines were oxidized.

The observations suggested that the entire precolumn was oxidized by the loading mobile phase in an irreversibly way. Even though the column was treated with sodium thiosulphate, after some injections the compounds were oxidized. Oxidizing agents in the mobile phase can oxidize Hypercarb columns. In the present study, fluorinated acids were used and these types of mobile phase additives have not been used by others.

With compounds that have electron-donating or –accepting properties, graphite forms reversible electrovalent compounds with simultaneous exchange of charge [43]:

$$\frac{n}{x}C_x + e^- \rightleftharpoons \frac{1}{x}\left[C_n^-\right]_x \qquad \text{electron-donor} \qquad (1)$$

$$\frac{n}{x}C_x \rightleftharpoons \frac{1}{x}\left[C_n^+\right]_x + e^- \qquad \text{electron-acceptor} \qquad (2)$$

Fluorinated acids are electron-accepting compounds and can thus form electrovalent compounds with graphite according to (2). The graphite gets a positive charge which corresponds to the observations by Shibukawa [37]. Since this process is reversible, it cannot be used to explain the behavior of the Hypercarb stationary phase material observed in the present study.

Graphite can react with oxidizing acids to form covalently or ionic bounded graphite-compounds. In these graphite-compounds, elements like fluorine or oxygen are deposited in the graphite forming electron-pairing bounds to carbon atoms [43]. Alternatively, graphite can be oxidized to ionic graphite salts by strong acids in the presence of oxidizing agents [43]. PFPA has a pK_a-value of 0.18. Dissolved oxygen in the mobile phase could be a potential oxidizing agent.

Both theories can be used to explain the irreversible oxidation of all catecholamines. The properties of the stationary phase material may have changed over the entire column. Because of the mentioned reactions of graphite, there may be no differences in oxidation behaviour in parts of the column as described in literature [36, 37]. The formation of a salt or a covalently bounded compound may be irreversible and this could explain why a column exposed to fluorinated acids never gained its original properties even though it was treated with reducing agents.

3.4.1 Prevention of oxidation

3.4.1.1 Considerations for loading mobile phase and trap column

Some precautions to prevent oxidation have been mentioned in chapter 3.2. To prevent the oxidation of the column by mobile phase additives, alternatives to both the loading mobile phase and the precolumn have been considered. Avoiding fluorinated acids in the loading mobile phase leaves pentanoic acid or benzoic acid as alternative since hydrophobic ion-pairing agents are needed to provide high enough retention. Since benzoic acid contains a benzene ring, it could possibly give high retention on Hypercarb. However, bad peak shape was obtained when benzoic acid was used as ion-pairing agent (figure 19). The pk_a of benzoic acid is similar to that of AcOH (4.20 vs. 4.79). One reason for the bad peak shape could be that AcOH could not exchange efficiently with the benzoic acid ion-pair, and this results in different retention on the analytical column for parts of the analyte. Pentanoic acid has a pk_a-value of 4.78, which is about the same as AcOH. Thus it was also anticipated that AcOH could not exchange with pentanoic acid in an ion-pair.

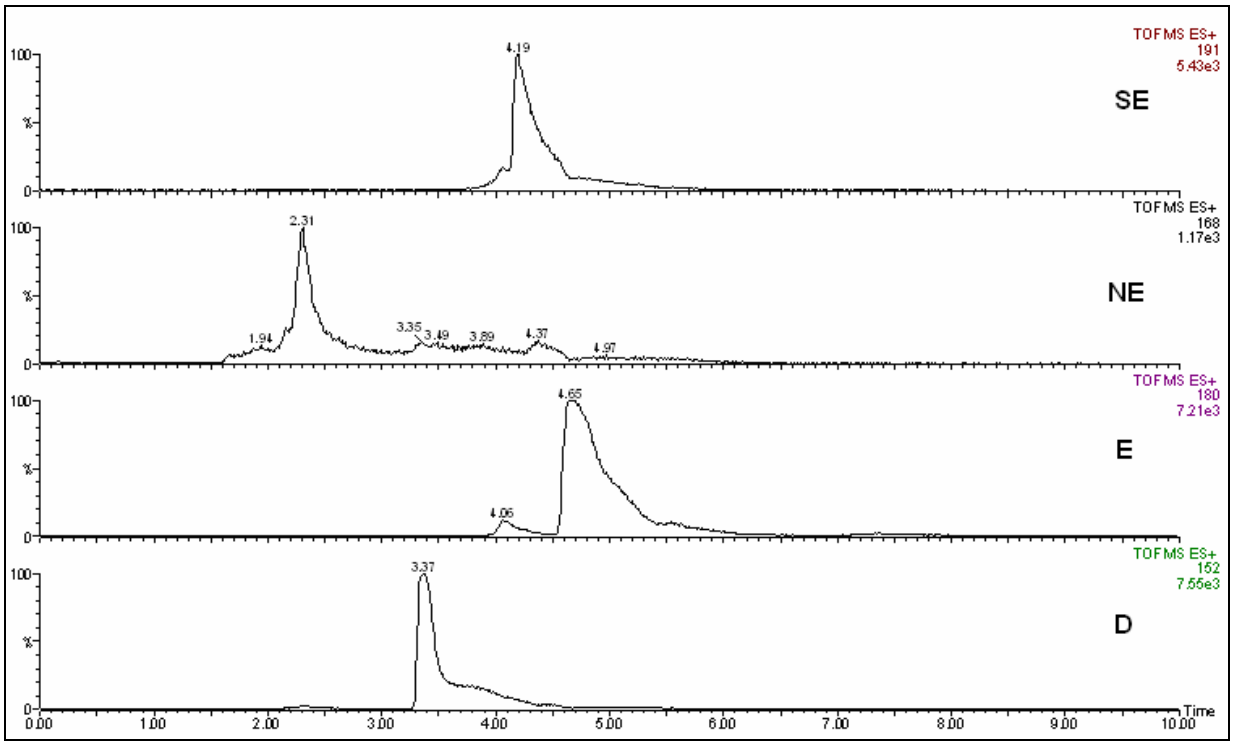


Figure 19: EIC of SE (*m/z* = 191), NE (*m/z* = 168), E (*m/z* = 180), D (*m/z* = 152) when 5 mM benzoic acid is used as ion-pairing agent in the loading MP. The SP of both columns was Hypercarb. A sample concentration of 250 ng/ml was used and the sample loop had a volume of 25 µl. The loading MP had a flow rate of 20 µl/min. The separation MP consisted of H_2O/AcN added 0.1%AcOH with a flow rate of 5 µl/min.

Hence, both an ion-exchange column and a packed PS-DVB column were considered as precolumn.

As shown by Storbråten [34], 10% acetonitrile was needed to get elution of the compounds from the ion-exchange column. Under these conditions, no refocusing on the Hypercarb analytical column would have been achieved for D, E, and NE, since dilution to the initial condition of the gradient is impossible. By diluting the eluate from the precolumn, bad peak shapes would have been obtained since 100% refocusing on the second column is not given.

The preconcentration of 0.1 mg/ml catecholamines on a packed PS-DVB column using H_2O + 0.1% HFBA as mobile phase and an injection volume of 50 nl was not successful.

3.4.1.2 Considerations of additives to stock solutions / mobile phase

Efforts were made to prevent oxidation of the catecholamines by adding anti-oxidants to either the stock solution or the separation mobile phase.

Ascorbic acid is a common reducing agent. It is reported that ascorbic acid interferes in voltammetric determinations of D [44], thus it might be easier oxidized than the catecholamines. Additionally, ascorbic acid is proposed to be added as antioxidant for sample

storage [45]. Thus, the stock solution was prepared in 0.1 M ascorbic acid to stabilize the sample. Working solutions of D, NE and SE were injected in the simplified system and no oxidation of the analytes was observed. SE and ascorbic acid have similar molecular masses and figure 20 shows that there was no peak overlapping when SE and a blank containing ascorbic acid are injected. No peak was observed in the blank injection, thus the ascorbic acid had been oxidized.

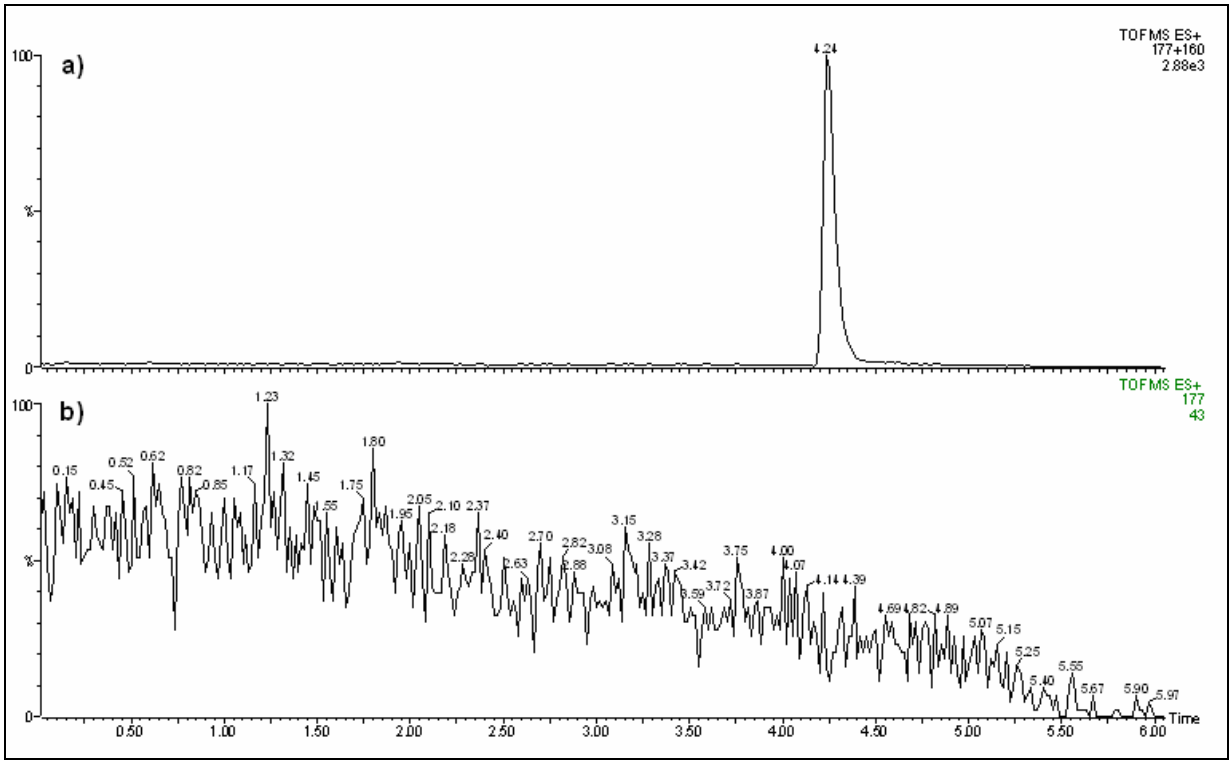


Figure 20: EIC of an injection of serotonin **(a)** and a blank containing ascorbic acid **(b)** on the Hypercarb analytical column. The MP consisted of H_2O/AcN added 0.1% AcOH with a flow rate of 5 µl/min. The injection volume was 50 nl and the concentration of serotonin was 0.1 mg/ml.

When a working solution containing all catecholamines was injected, oxidation of D was observed in the 2nd injection (figure 21a and b). A working solution containing D was injected to confirm oxidation of the compound (figure 21c and d). The working solution was injected with the column disconnected to see if oxidation had occurred in the solution, but no oxidation was observed (figure 21e). This suggested that the ascorbic acid was oxidized leaving the catecholamines in their reduced form in the first couple injections, but when several injections were performed the amount of ascorbic acid added to the stock solutions was not enough to prevent oxidation of the catecholamines. 0.1 mmol ascorbic acid was added to the A reservoir (100 ml) of the separation mobile phase. With ascorbic acid added to the mobile phases SE could not be detected since the mass of ascorbic acid $[M+H]^+$ interfered with the mass of SE $[M+H]^+$. Thus, ascorbic acid was not suitable for use as mobile phase additive to prevent oxidation. The addition of ascorbic acid to the loading mobile phase was not tried.

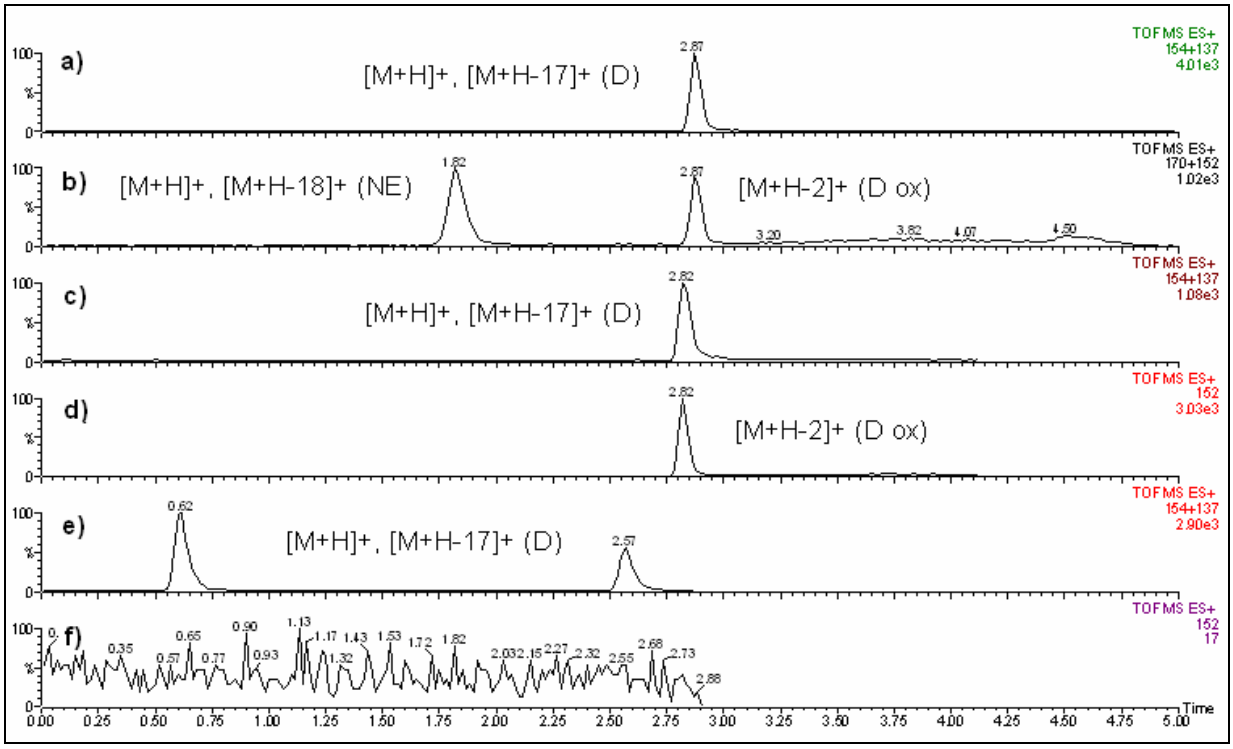


Figure 21: EIC showing the oxidation of D on the Hypercarb analytical column with ascorbic acid added to the stock solution. The MP consisted of H_2O/AcN added 0.1% AcOH with a flow rate of 5 µl/min and the injection volume was 50 nl. **a,b)** injection of a 0.1 mg/ml solution catecholamines, *m/z* = 137+154 (**(a)** D), *m/z* = 152 (**(b)** fragment of NE, oxidation of D); **c,d)** injection of a 0.1 mg/ml solution D, *m/z* = 137+154 **(c)**, *m/z* = 152 (**(d)** oxidation of D); **e,f)** injection of a 0.1 mg/ml solution D with the column disconnected, *m/z* = 154 **(e)**, *m/z* = 152 **(f)**.

Inorganic reducing agents have smaller molecular masses than organic anti-oxidants. Therefore, interference with the analyte masses will not take place. Additionally, they ideally have no retention on the column. A common inorganic reducing agent is sodium thiosulphate. Törnkvist et al. [36] demonstrated the reduction of oxidized Hypercarb stationary phase with sodium thiosulphate. Alternatively, salts of the sulfurous acid, sulphite (SO_3^{2-}) and bisulphite (hydrogensulphite, HSO_3^-) are powerful reducing agents and they are used to stabilize samples (see chapter 1.1). However, reducing power is lost in acidic solution since under acidic conditions primarily sulfur dioxide (SO_2) exists [43, 46]. None of the research groups using MS detection have used sulphite or bisulphite as anti-oxidant. Thus, in this study sodium thiosulphate was chosen as reducing agent.

1 mmol sodium thiosulphate was added to the A reservoir of the mobile phase (100 ml). The use of thiosulphate resulted in signal suppression and clogging of the capillaries rapidly occurred, because thiosulphate is not enough soluble in AcN. Thus it was not an alternative to add sodium thiosulphate to the mobile phase.

3.4.1.3 Improvement of the system

Shibukawa and co-workers demonstrated that a cobalt-trans-1,2-diaminocyclohexanetetraacetate (Co-DCTA) complex was oxidized on the first of two Hypercarb columns coupled in series [26]. Therefore, guard columns were coupled between the pumps and the valves (figure 2). They were oxidized by the mobile phase first, lengthening the life of the reduced precolumn and analytical column. Figure 22 shows the average retention times of the analytes during validation of the method. Three replicates of validation solutions at three concentration levels were injected. Including a blank at the beginning of each day, the number of injections performed was 10. The retention times were nearly constant. This indicates that by adding the guard columns the method did not suffer from severe oxidation for at least 10 injections.

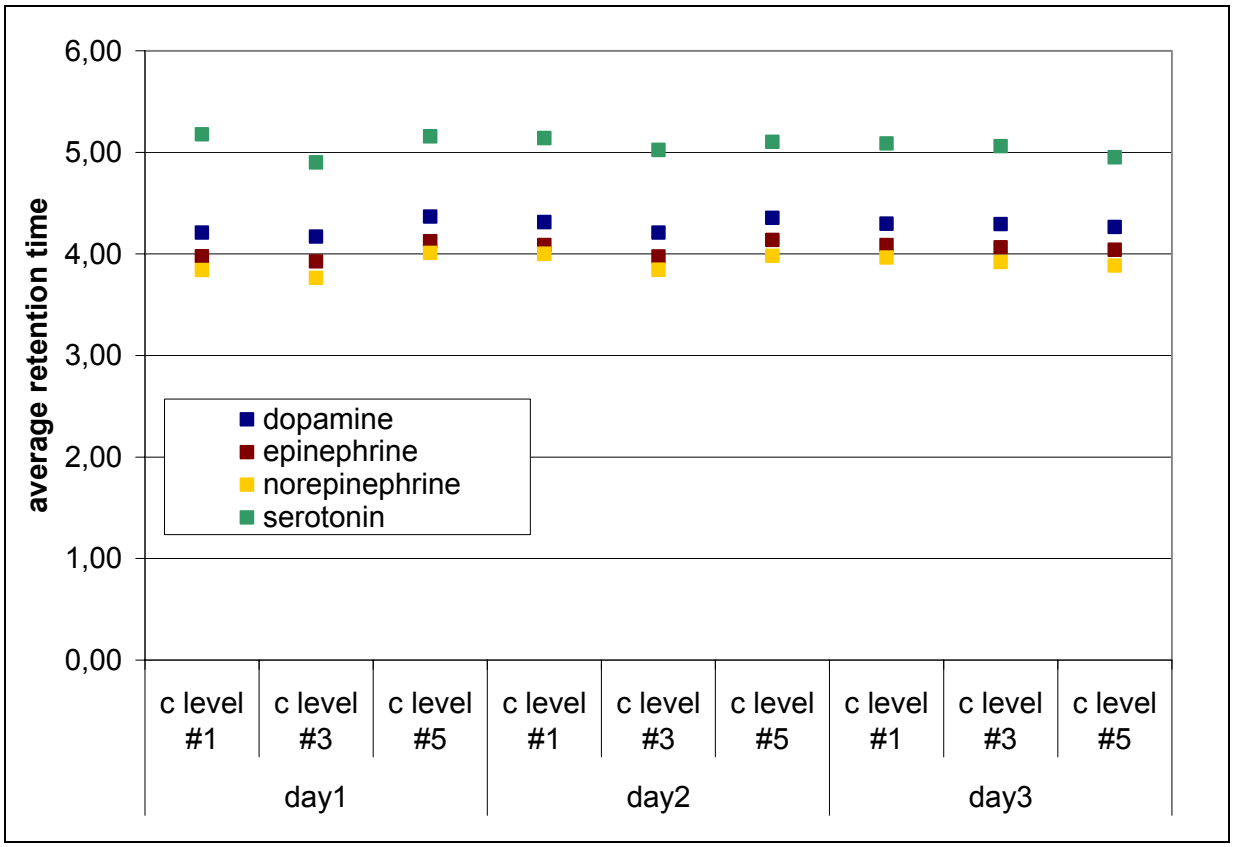


Figure 22: Average retention times of analytes during validation when three replicates of validation solutions at three concentration levels were injected.

Figure 23 shows the peak areas of E in validation solution #5 when only *m/z* = 166+184 (protonated molecular ion + fragment ion) is included ("E") versus the peak areas when the mass of oxidized E is taken into account as well (*m/z* = 166+184+182+180, "E(ox)"). As can be seen from the figure, the increase in peak area when oxidation is taken into account is not high. Calculations (appendix table A7) show that a maximum of 10% higher peak area is obtained. Again, this indicated that the method did not suffer from severe oxidation for at least 10 injections. Thus, oxidation was treated as negligible during validation. The pattern for E(ox) was the same as for E, thus neither the within- nor the between-day precisions were affected by oxidation.

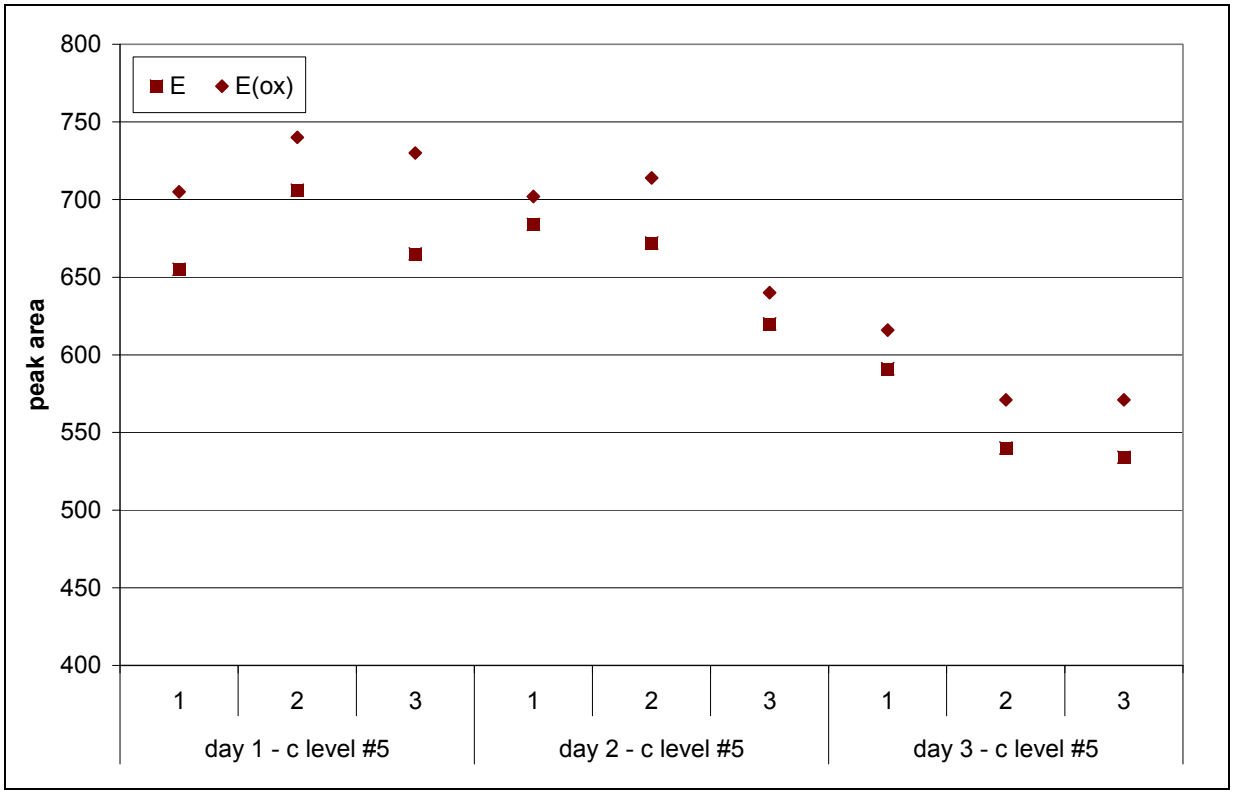


Figure 23: Plot of the peak area of validation solution #5 for E (*m/z* = 184+166) and E(ox) (*m/z* = 184+166+182+180).

To regain their original properties, the guard columns were treated with 1 M sodium thiosulphate at the end of each day (once they were oxidized). Figure 24 shows the EIC of NE and D before and after the treatment of the column with thiosulphate. Oxidized D can be seen in the EIC of NE before treatment with thiosulphate, since the fragment of NE ($[M+H-18]^+$) and oxidized D have the same *m/z*-values (*m/z* = 152). After the treatment with thiosulphate no oxidation was observed.

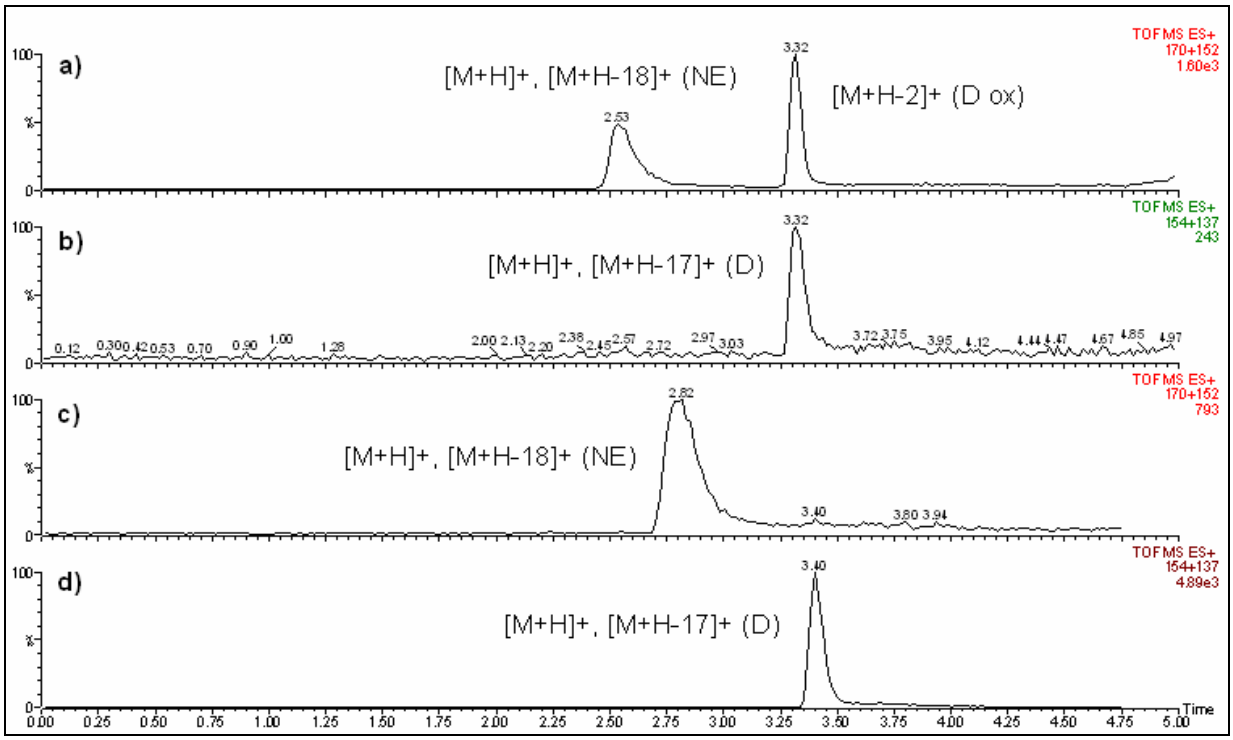


Figure 24: The effect of flushing with sodium thiosulphate. **a)** EIC of NE+NE-18 before flushing, oxidation of D is observed; **b)** EIC of D+D-17 before flushing; **c)** EIC of NE+NE-18 after flushing; **d)** EIC of D+ D-17 after flushing.

Since no alternative to the fluorinated acid PFPA as ion-pairing agent was found, the method was validated using the loading mobile phase described in chapter 3.3.2. The mobile phase described in chapter 3.3.1 was used as separation mobile phase since no suitable additives were found to prevent oxidation.

3.5 Injection volume

When the concentration of the analytes in the sample is low, as it is expected for catecholamines in brain samples, high injection volumes are desirable to enhance sensitivity. A problem with large volume injections is column overload and breakthrough.

Injection volumes of 25 µl, 50 µl, 100 µl and 200 µl were tested. A 250 ng/ml solution was used as injection solution. Because of the high concentration, peak saturating occurred and thus the peak areas of the C_{13}-isotopes were used to find the optimal loop volume. Figure 25 shows the peak area plotted against the loop volume for each catecholamine. The area was found from an average of 2 (100 µl) or 3 injections.

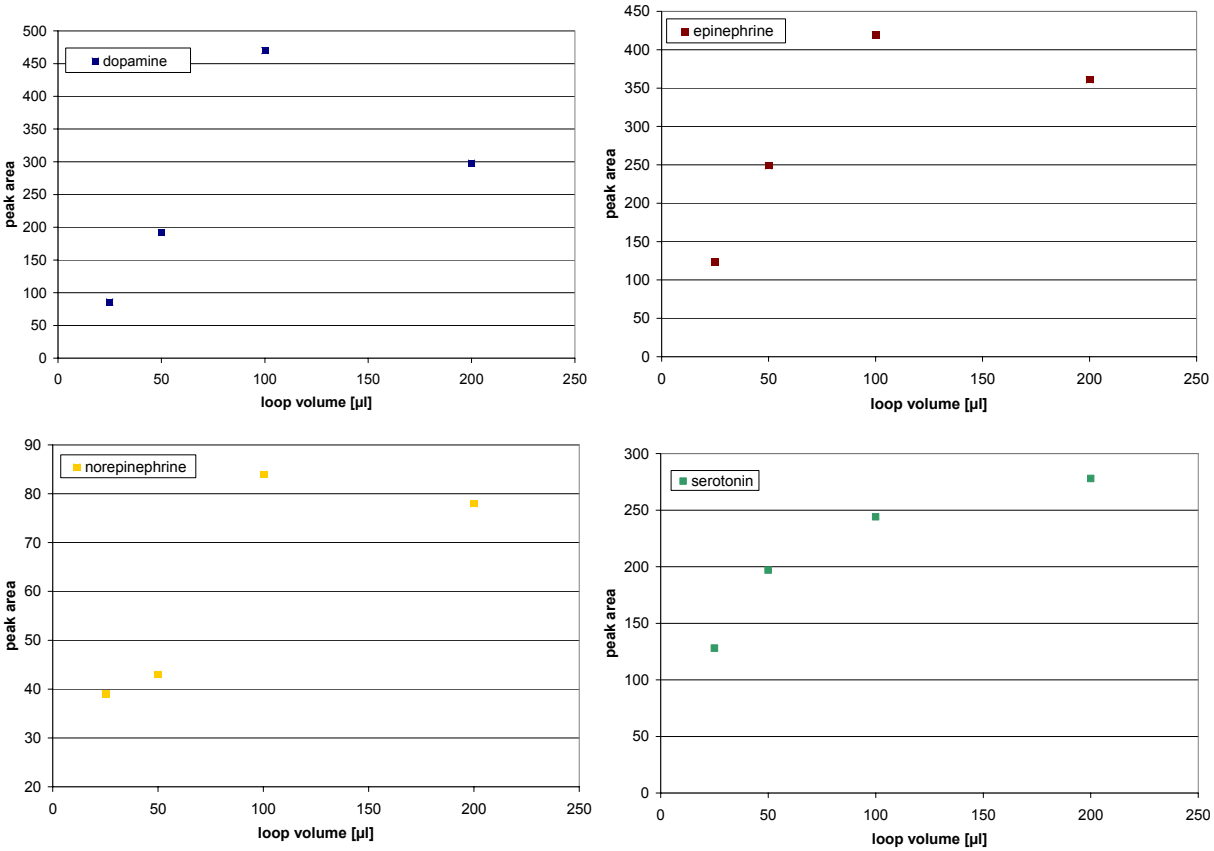


Figure 25: Variation of the peak area of the C_{13}-isotopes with different loop sizes.
The SP of both columns was Hypercarb. A sample concentration of 250 ng/ml was used. The loading MP consisted of H_2O+0.1% PFPA with a flow rate of 50 µl/min. The separation MP consisted of H_2O/AcN added 0.1% AcOH with a flow rate of 5 µl/min.

The peak area of D, E, NE and SE increased when the injection volume was increased from 25 µl to 50 µl and to 100 µl. A further increase of the injection volume resulted in a decrease of the peak area of D, E and NE, while a smaller increase of the peak area was observed for SE. To be sure to avoid column overload of D, E and NE, an injection volume of 100 µl was chosen as maximum.

3.6 Robustness

In a column-switching system several parameters can be varied to achieve high sensitivity and each of them has to be optimized. Parameters tested in this work were the loading flow rate; the reconditioning time of the precolumn with the loading mobile phase; the loading time of the sample on the precolumn; delay-time of the gradient e.g. the time between gradient start at the pump and switching time of valve II from the load to the inject position to start

elution from the precolumn; use of acetonitrile from three different producers; degassing of the mobile phase after preparation versus on-line degassing of the mobile phase during analysis.

The guard column for the loading mobile phase (guard column II) had to be changed after the loop size was determined. This change resulted in an increase of the backpressure in the system, and it was not possible to operate with a loading flow rate of 50 µl/min. A loading flow of 25 µl/min had to be used in the final system.

The delay-time was first evaluated. Figure 26 shows a chromatogram of an acquisition where the valve was switched at the same time as the gradient was started (no delay-time) and a chromatogram of an acquisition where the valve was switched 3 min after the gradient was started (3 min delay-time). The intensity of the NE signal is approximately 8 times lower and the peak is broader in the first case. NE is not entirely focused on the precolumn at the starting conditions of the gradient (0% B). Therefore, when the valve is switched at the same time as the gradient is started, NE is slowly eluted from the column. The delay-time of the pump, e.g. the time it takes the mobile phase to get from the pump to the column is approximately 3 min. Waiting 3 min thus improves the NE signal and peak shape since it is eluted by the gradient.

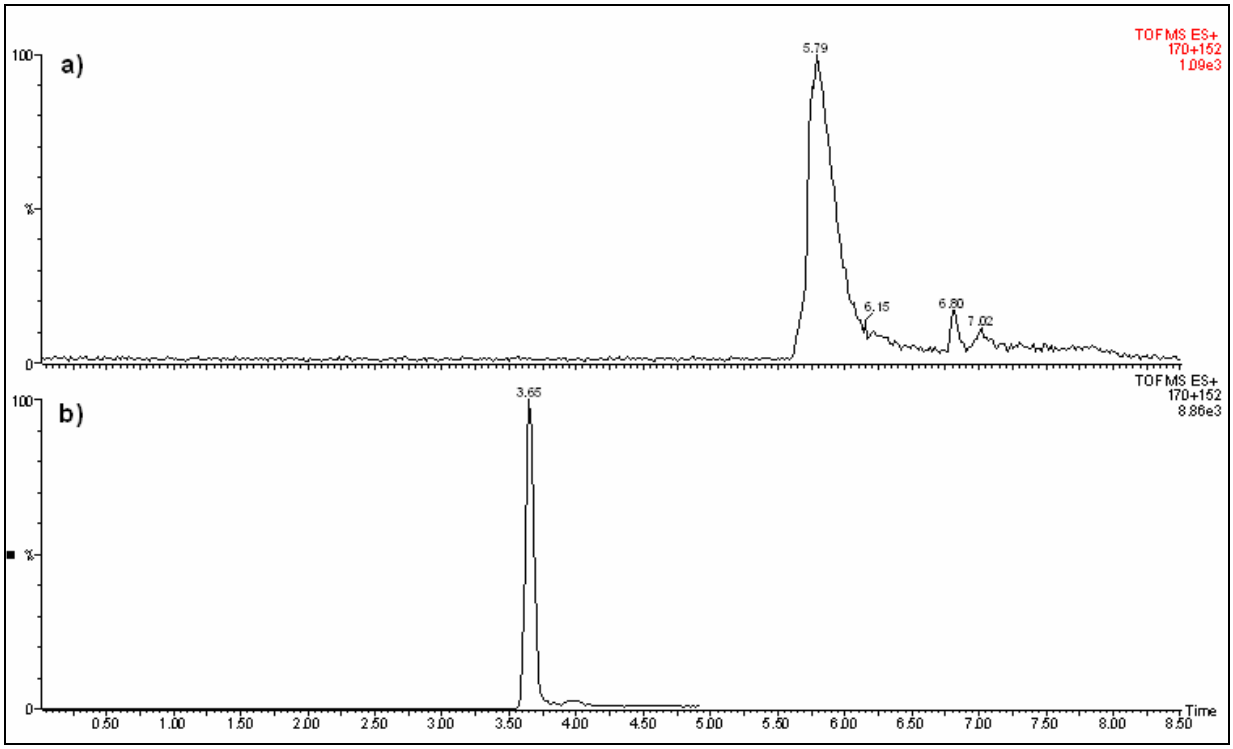


Figure 26: EIC showing differences in the peak shape and the intensity for the norepinephrine peak at **a)** no delay-time; **b)** 3 min delay-time.
The SP of both columns was Hypercarb. A sample concentration of 250 ng/ml was used and the sample loop had a volume of 100 µl. The loading MP consisted of H_2O+0.1% PFPA with a flow rate of 25 µl/min. The separation MP consisted of H_2O/AcN added 0.1% AcOH with a flow rate of 5 µl/min.

All remaining tests were performed using a 3 min delay time and 25 µl/min loading flow. Figure 27 shows the peak areas plotted against different reconditioning times of the precolumn and different sample loading times.

At first, the loading time was held constant at 4 min. The reconditioning time of the precolumn was set to 2 min in the first to runs. It was then increased to 3 min in the next two runs. Due to peak saturating, the peak area for D was calculated using C_{13}-isotopes. The peak area increased except for NE. There is no good explanation why the peak area of NE decreased when the reconditioning time is increased by 1 min, since a decrease is also observed when oxidation is taken into account. Despite this fact, a reconditioning time of 3 min was chosen. There after, the loading time was increased to 5 min, holding the reconditioning time at 3 min. In this case, an increase in the peak area was observed for all four analytes. Thus, the reconditioning time of the precolumn was chosen to be 3 min and the loading time 5 min.

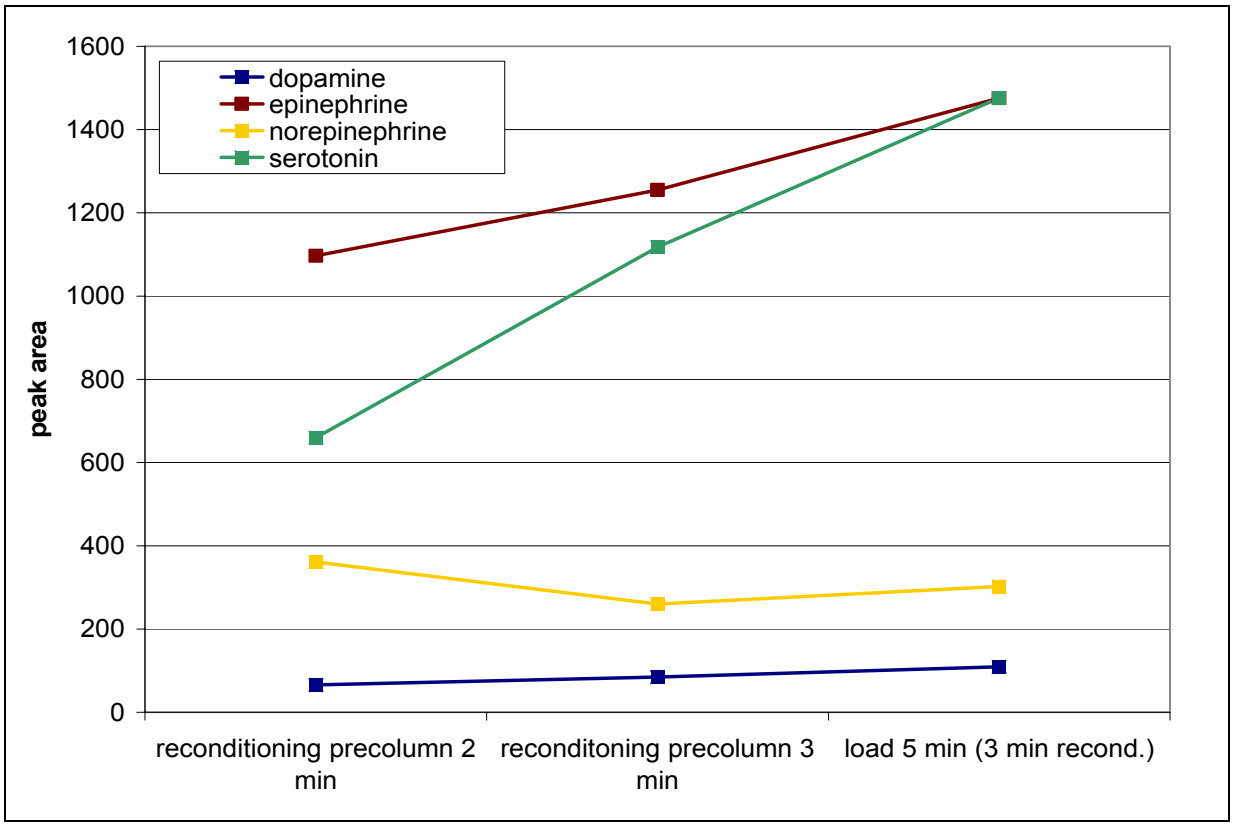


Figure 27: Variation of peak area with different reconditioning and loading times, D: *m/z* = 155+138. The SP of both columns was Hypercarb. A sample concentration of 62.5 ng/ml was used and the sample loop had a volume of 100 µl. The loading MP consisted of H_2O+0.1% PFPA with a flow rate of 25 µl/min. The separation MP consisted of H_2O/AcN added 0.1% AcOH with a flow rate of 5 µl/min.

Acetonitrile from three different producers was tested. No differences in oxidation behavior were observed.

The mobile phase was degassed with He after preparation for around 15 min. On-line degassing with He did not increase the number of injections possible before thiosulphate treatment was needed.

3.7 Validation

A representative EIC for the simultaneous determination of four catecholamines using the developed column-switching method is shown in figure 28. A somewhat poorer resolution compared to the separation on the simplified system (figure 5) was obtained because of different lengths of the analytical columns.

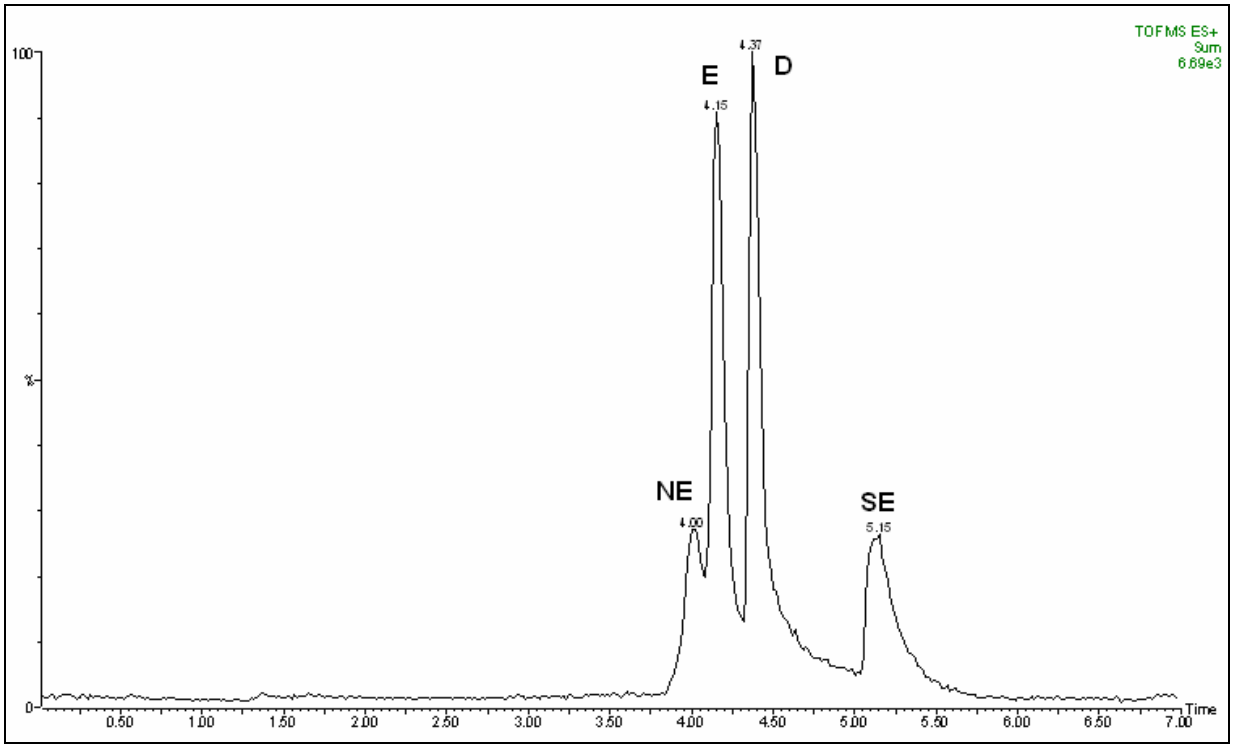


Figure 28: EIC (sum) of a separation of four catecholamines with the developed column-switching method.
The precolumn was a 25 x 0.32 (i.d.) mm 5 µm Hypercarb column and the analytical column was a 34 x 0.32 (i.d.) mm 5 µm Hypercarb column. A sample concentration of 50 ng/ml (D,E) and 60 ng/ml (NE, SE) was used and the sample loop had a volume of 100 µl. The loading MP consisted of H_2O+0.1% PFPA with a flow rate of 25 µl/min. The separation MP consisted of H_2O/AcN added 0.1% AcOH with a flow rate of 5 µl/min.

The validation was carried out using the validation solutions described in table 5.

3.7.1 Limit of detection and limit of quantification

The limit of detection (LOD) for each catecholamine was determined by the concentration giving a signal to noise ratio (S/N) of 3, and the limit of quantification (LOQ) giving S/N = 10.

The limits of detection for dopamine, epinephrine, norepinephrine and serotonin for a 100 µl injection volume were 1.5, 0.75, 3.0 and 1.5 ng/ml, respectively. Values of the mLOD, cLOD, mLOQ and cLOQ are given in table 10. Compared to other methods with (ESI-) MS detection that are described in the literature, better limits of detection were performed in the present study.

Table 10: Limit of detection and limit of quantification. Injection volume: 100µl; loading flow: 25µ/min.

	Dopamine	*Epinephrine*	*Norepinephrine*	*Serotonin*
mLOD [ng]	0.15	0.075	0.30	0.15
cLOD [ng/ml]	1.5	0.75	3.0	1.5
mLOQ [ng]	0.75	0.30	1.0	1.0
cLOQ [ng/ml]	7.5	3.0	10	10

Peterson et al. [4, 8] described a CE system with ESI-TOF detection. In their work they get detection limits of 45.9 ng/ml, 18.8 ng/ml, 50.7 ng/ml and 26.4 ng/ml for D, E, NE and SE, respectively. These detection limits are much higher (about 17 to 30 times) than the limits of detection obtained in this work. Another work using mass spectrometry detection was done by Chan and co-workers [3] and they obtained limits of detection of 5 ng/ml for D, E and NE, which is slightly higher than presented here. Törnkvist and co-workers use µLC with ESI-MS detection and got limits of detection of 0.5 ng/ml and 1.5 ng/ml for NE and D, respectively [16].

With electrochemical detection lower limits of detection can be obtained. He et al. presented a LC-EC system for determination of NE and E [15] with limits of detection of 12 pg/ml for both compounds. Niwa and co-workers obtained a limit of detection of 1 pg/ml for D with their LC-EC system [14].

3.7.2 Linearity

To check the linearity one of the replicates of each validation solution was randomly chosen from the validation experiment. Figure 29 shows a first series of linearity curves for D, E, NE and SE. Linear and second degree polynomial regression lines were calculated. All correlation coefficients (R^2-values) are given in table 11. D and E have good linear fit, although the polynomial fit is even better. NE and SE have poorer linear fit than D and E. The correlation is

greatly improved with the polynomial fit. Thus, the curves for both NE and SE are second-degree polynomial.

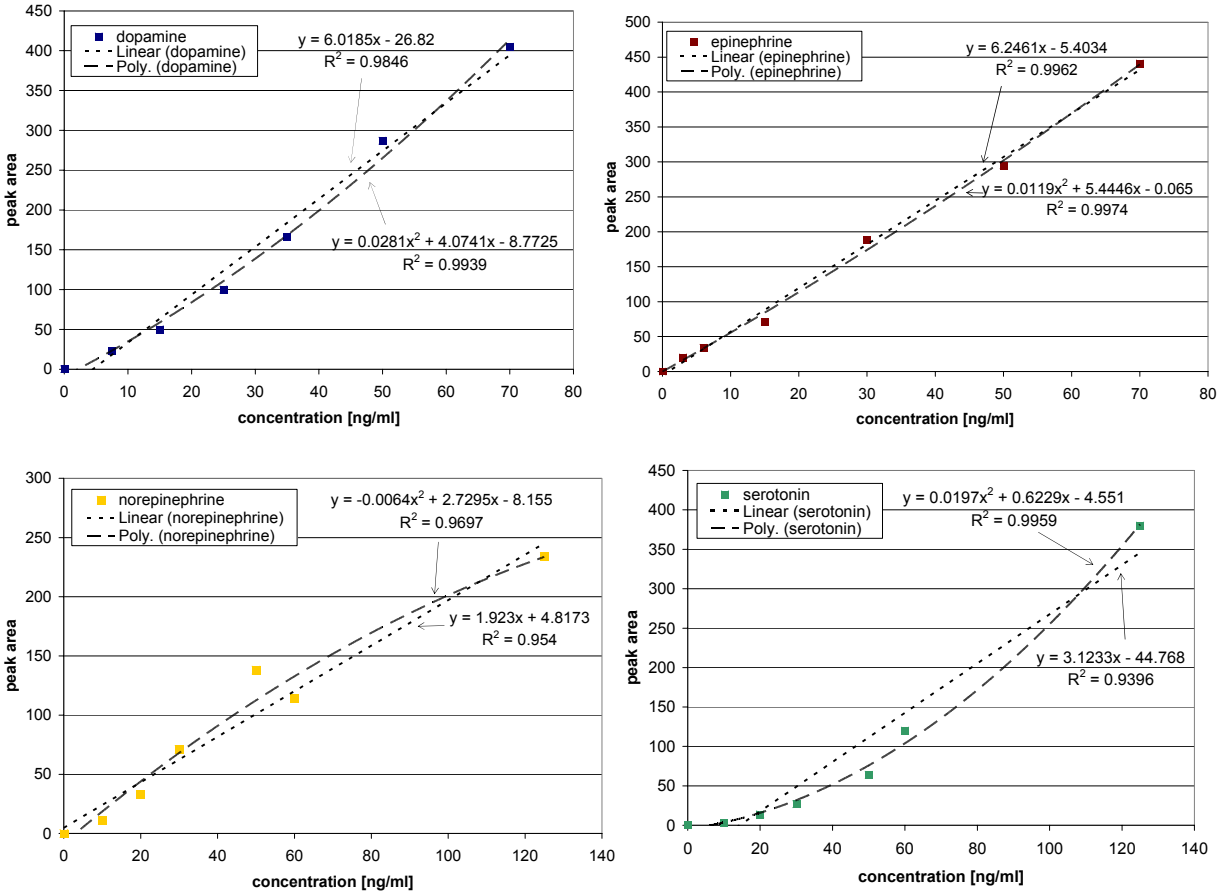


Figure 29: Linearity curves for D, E, NE and SE using a TDC stop voltage of 100 mV. Data points are chosen randomly from three replicates of validation solution #1, #3 and #5.

Table 11: Correlation coefficients for the linear and the polynomial fit at both 100 mV and 50 mV TDC-stop voltage.

TDC-voltage	*R^2-values*	*Dopamine*	*Epinephrine*	*Norepinephrine*	*Serotonin*
100 mV	*linear*	0.985	0.996	0.954	0.940
	polynomial	0.994	0.997	0.970	0.996
50 mV	*linear*	0.992	0.997	0.985	0.998
	polynomial	0.998	0.997	0.999	0.999

Solbu [38] described a similar problem in his work. He obtained a linear curve by reducing the standard TDC stop voltage of the mass analyzer. The first series of linearity curves was obtained using a TDC stop voltage of 100 mV. In a second series, the TDC stop voltage was set to 50 mV (figure 30). Validation solutions #4 and #6 were not included in this series. All catecholamines showed an improved linearity, however all polynomial fits were better and especially the curve of NE still looks polynomial. The better polynomial fit could be explained

by the fact that a polynomial curve always will fit a linear curve. Since better linearity and better sensitivity was obtained with a TDC-stop voltage of 50 mV, the series on day 2 and day 3 were measured with this voltage.

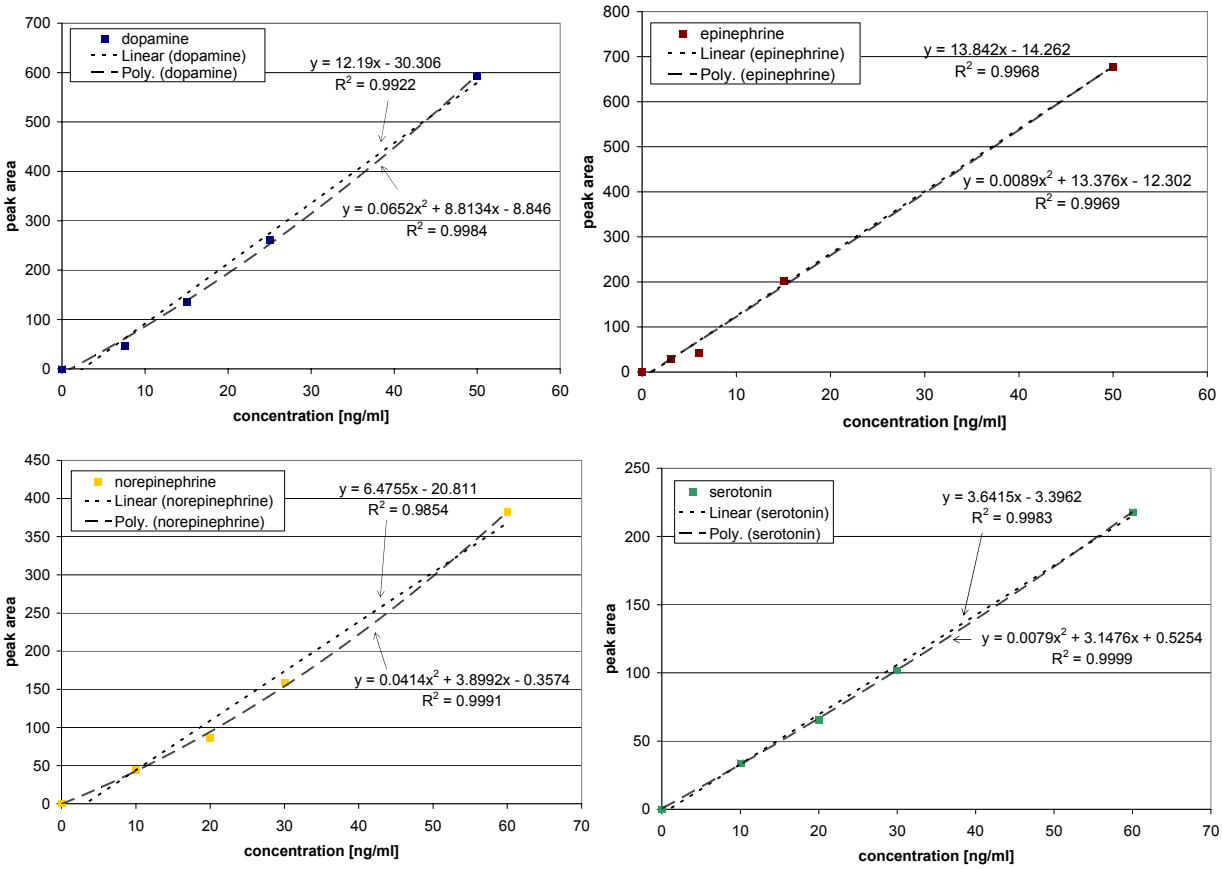


Figure 30: Linearity curves for D, E, NE and SE using a TDC stop voltage of 50 mV. Data points are chosen randomly from three replicates of validation solutions #1, #3 and #5.

3.7.3 Repeatability

The within-day and between-day precision of the retention times and the peak areas were determined on three different days using three replicates at three concentration levels. They were calculated by analysis of variance (ANOVA, appendix, chapter 6.3.3.2). In addition, the within-day precision for each concentration level on each day was calculated by Microsoft Excel in the conventional way (appendix, tables A8-A9). All calculations were performed using the series of concentration levels measured with a TDC-stop voltage of 50 mV.

Table 12 shows the within-day and between-day repeatability of the retention times calculated by ANOVA. The within-day and between-day precision of the peak areas calculated by ANOVA is given in table 13. All values are given as % relative standard deviation (% RSD). Raw-data are given in the appendix in tables A5-A6.

Table 12: The within- and between-day precisions of the retention times calculated by ANOVA.

	Concentration level (table 5)	*Dopamine RSD [%]*	*Epinephrine RSD [%]*	*Norepinephrine RSD [%]*	*Serotonin RSD [%]*
Within	1	2.7	3.0	5.9	3.8
	3	2.3	2.6	3.0	2.1
	5	0.7	0.7	1.0	1.0
Between	1	2.2	2.7	1.2	1.5
	3	2.6	3.0	3.5	2.9
	5	2.2	2.2	2.9	3.7

The repeatability of the retention times is acceptable.

Table 13: The within- and between-day precisions of the peak areas calculated by ANOVA.

	Concentration level (table 5)	*Dopamine RSD [%]*	*Epinephrine RSD [%]*	*Norepinephrine RSD [%]*	*Serotonin RSD [%]*
Within	*1*	10	6.6	9.0	58[#]
	3	6.7	11	11	11
	5	8.3	4.8	6.2	13*
Between	*1*	15	29	26	72[#]
	3	8.0	21	8.4	25
	5	17	18	53	41*

* A value that was close to be rejected by Grubbs test is included. [#] Integration of concentration level #1 was difficult for serotonin due to no clear peaks.

The within-day precisions of the peak areas of D, E and NE are satisfactory for all concentration levels. A high value for the within-day precision of SE at the lowest concentration level was found. At this concentration level the peaks were low and difficult to integrate. It was assumed this was because the injections of validation solution #1 were performed first each day and that the system needed more time for reconditioning.

Generally, much higher values were obtained for the between-day precisions. The between-day precisions were, however, acceptable for all concentration levels of D and E. Slightly higher values were observed at the lowest and third concentration level of E, but no big differences between the days are apparent from the plot of all peak areas (figure 31).

The value at the highest concentration level of NE is too high. This can be explained by the fact that the peak areas of NE were much higher on the first day than on the next two days for the actual concentration level (figure 31).

The values at the lowest and highest concentration level of SE are too high. An explanation for the high value at the lowest concentration level might be difficulties of integration (see within-day precision). At the highest concentration level of SE a possible outlier (see appendix, chapter 6.3.3.1) was included in the first day. The actual values are marked with *. This might be the reason for the high values at this concentration level both for the within- and the between-day precision. A slight increase in peak areas from day 1 to day 3 in concentration level 5 can be seen for SE, which also might be responsible for the high between-day variation.

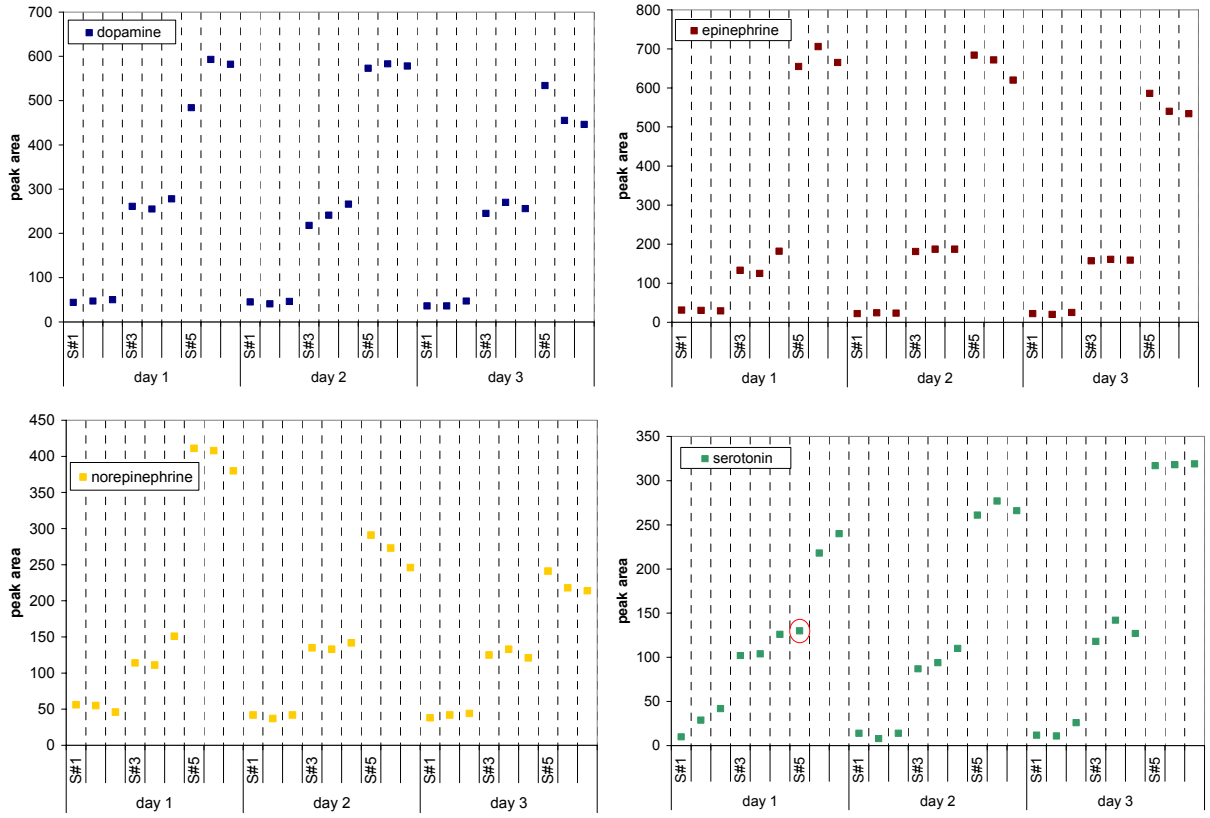


Figure 31: Plot of the peak areas of all concentration levels during validation. The value marked with a red ring is a possible outlier.

As can be seen from the between-day linearity test (appendix, figure A9), the peak area values of the fifth concentration level varied much more than in the other concentration levels. This fact cannot be explained by oxidation towards the end of a day, since as discussed in chapter 3.4.1.3 the pattern for E(ox) was the same as for E (figure 23) and thus the precisions are not affected by oxidation. Variations in the MS detection might be responsible for the high variation at this concentration level.

4. Conclusion

The present study has shown that the developed column-switching μLC-method coupled to ESI-TOF-MS detection has the capability of providing fast and simultaneous determination of dopamine, epinephrine, norepinephrine and serotonin with limits of detections that are lower or at the same level as reported before. The analytes were separated in less than 5 minutes on a Hypercarb column and the time of one injection including reconditioning and loading times was 22 min. The Hypercarb phase material has both advantages and limitations and precautions have to be taken when this material is used. Not all processes observed in this work involving the oxidation of the stationary phase and the analytes are understood. Further work should include investigation of the cause of oxidation by fluorinated acids and improvement of the method to obtain even better repeatability to analyze real samples (waste-fractions) from rat brain with high sensitivity.

5. References

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6. Appendix

6.1 Preliminary experiments

6.1.1 Testing of five different reversed-phase columns as separation columns for peptides

Five different reversed-phase analytical columns were tested using a mixture containing 0.1 mg/ml of angiotensin II, bradykinin and neurotensin (all obtained from Sigma-Aldrich).

The columns tested are shown in table A1. The Poroshell columns were obtained from Agilent Technologies, the Kromasil and Prontosil columns from G&T Septech AS (Kolbotn, Norway). The ACE column was packed in-house according to the procedure described in chapter 2.3. Stationary phase material was purchased from Mac-Mod Analytical Inc. (Chadds Fort, PA, USA).

Injections were performed with a Valco ChemInert model C4 injection valve equipped with a 50 nl internal loop. A 25 µl laboratory syringe from SGE was used as injection syringe. The column was kept in a Mistral oven (Spark Holland, Emmen, The Netherlands). The mobile phase was delivered by a Hitachi L-7110 (Merck) isocratic LC pump and detection was performed using a Spectra 200 UV-ViS detector (Spectra-Physics, San Jose, CA, USA) equipped with an on-column optical cell built in-house. The detection wavelength was set to 210 nm. The data processing unit was a Shimadzu C-R6A Chromatopac integrator.

Table A1: Reversed-phase analytical columns tested.

Type	*i.d. [mm]*	*Length [cm]*	*Pore size [Å]*	*Particle size [µm]*
(1) Poroshell 300 SB-C18	0.3	7.5	300	5
(2) Kromasil C18-100	0.3	5	100	3.5
(3) ProntoSIL C18-300	0.3	5	300	3
(4) Poroshell 300SB- C18	0.5	15	300	5
(5) ACE	0.32	10.1	100	3.5

The columns were tested with different compositions of water/AcN mobile phases, all added 0.1% TFA. Additionally, the efficiency was checked using toluene as test substance. The first three of those columns were tested at higher flow rates as well. Figure A1 shows a representative chromatogram from the test of the reversed-phase analytical columns.

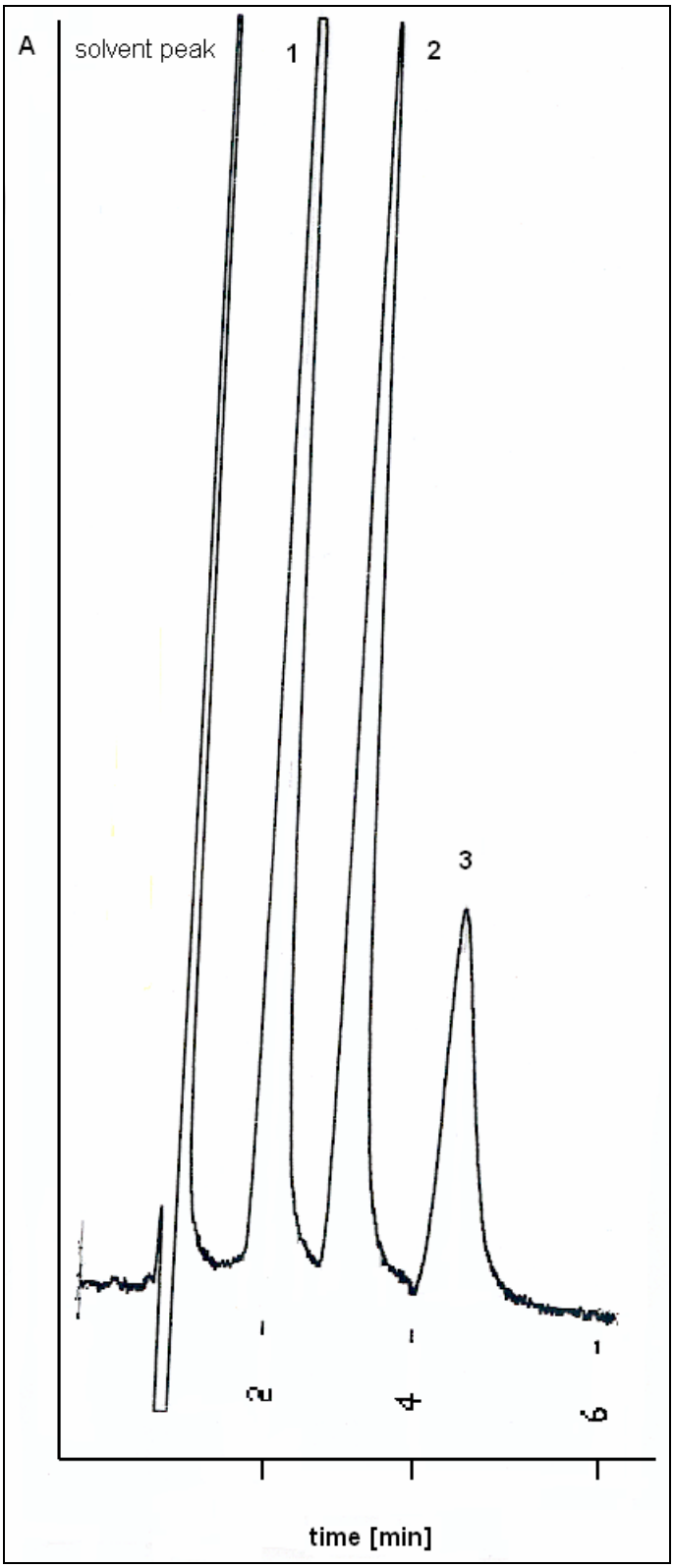

Figure A1: A representative chromatogram from the testing of reversed-phase columns. The column used was a Kromasil C_{18} column (500 x 0.3 (i.d.) mm, 3.5 µm). The MP consisted of 25% AcN and 0.1% TFA and had a flow rate of 5 µl/min. 50 nl of a solution containing 0.1 mg/ml angiotensin II, bradykinin and neurotensin was injected. Detection was performed at 210 nm.

A number of chromatographic parameters were established such as the k-value for the second peak, the plate number per meter, the selectivity, the asymmetry and the resolution between two peaks. Figures A2 to A8 present the obtained data.

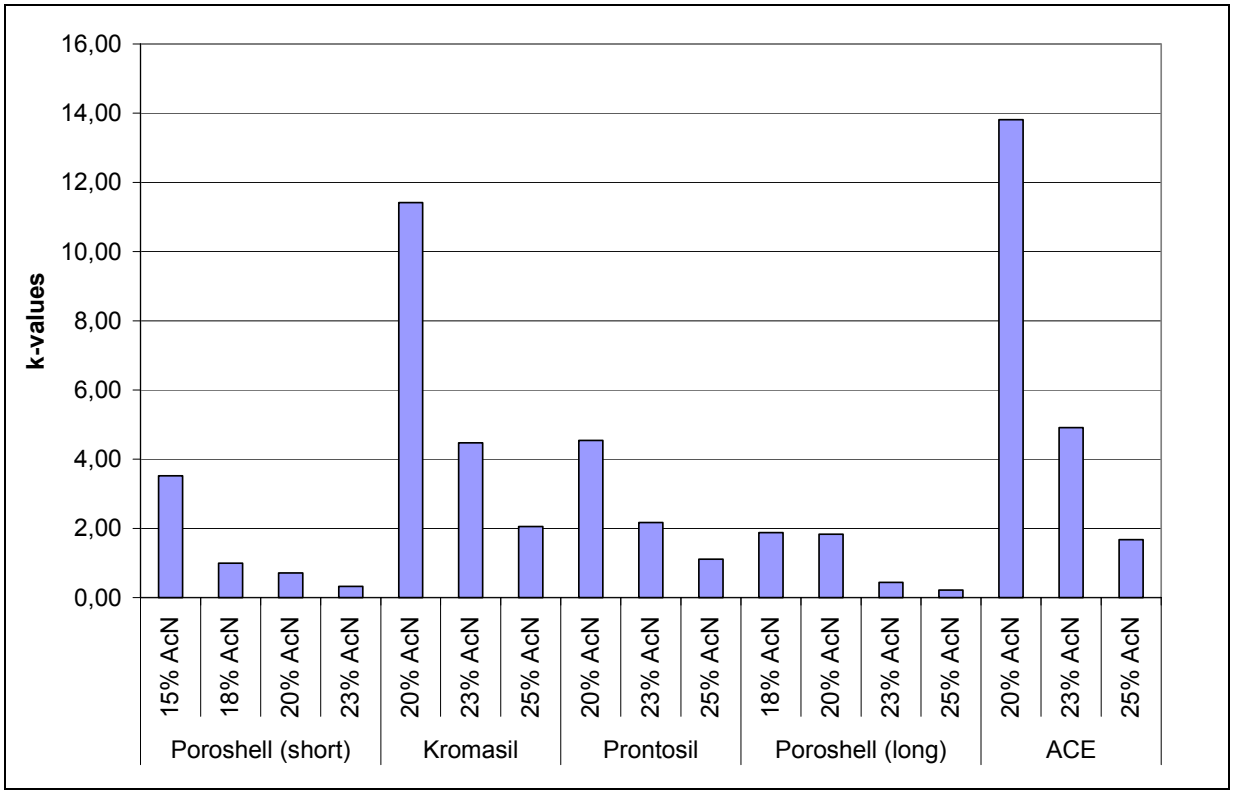

Figure A2: k-values of the second peak.

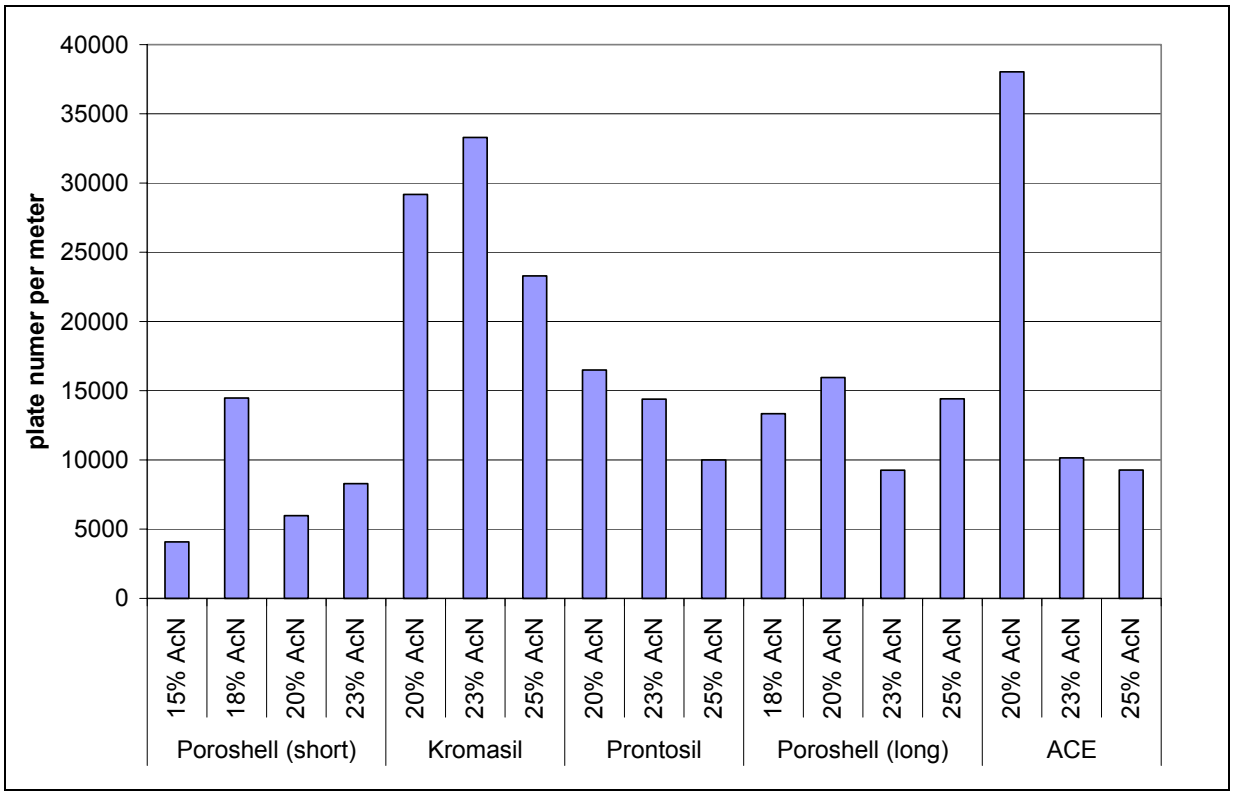

Figure A3: Plate number per meter at different MP compositions with a flow rate of 5 µl/min.

Figure A4: Selectivity between peak 1 and 2, and between peak 2 and 3 at different MP compositions.

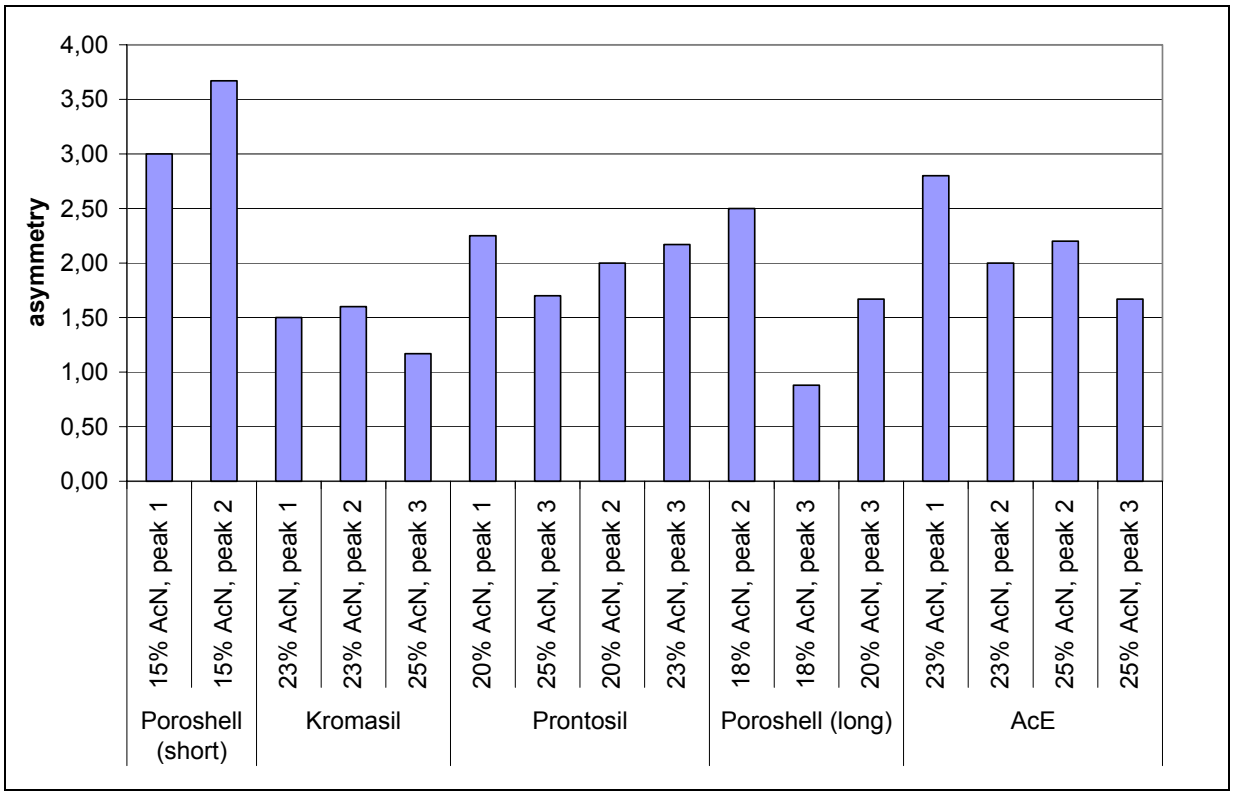

Figure A5: Asymmetry of peaks with k-values of 2-5.

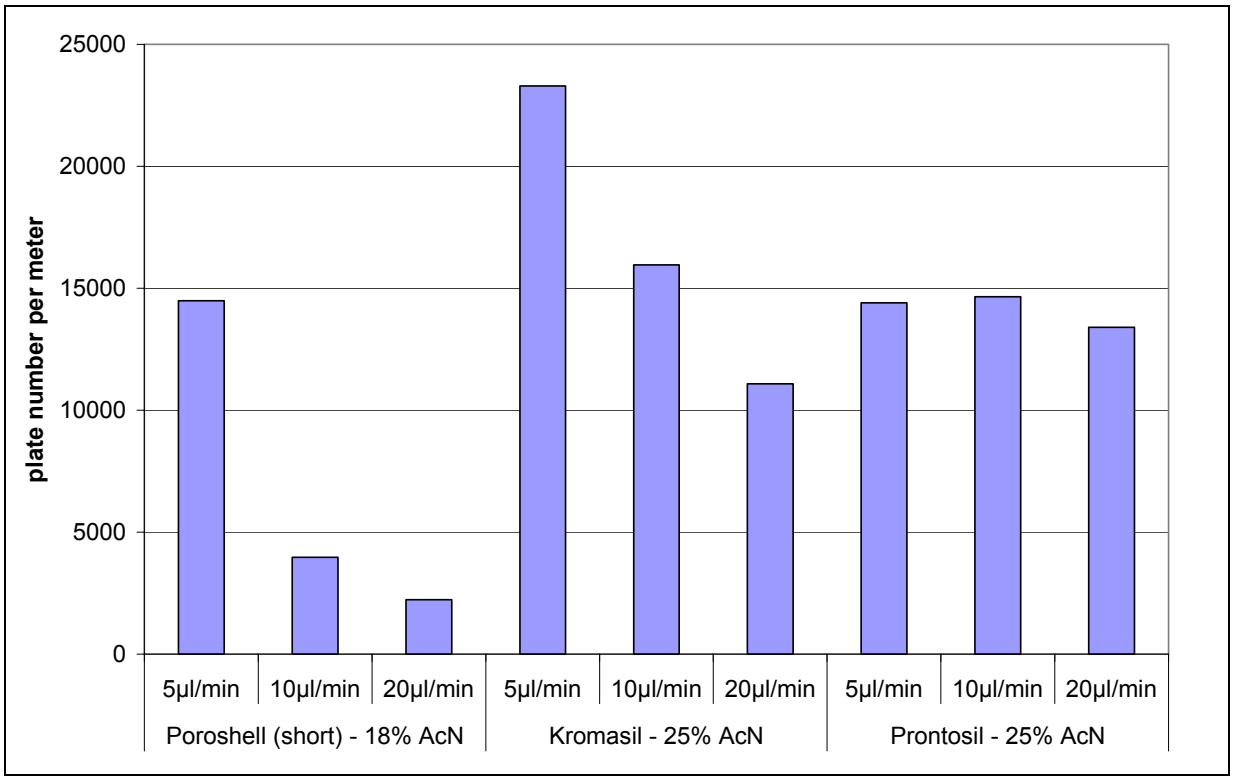

Figure A6: Plate number per meter at different flow rates.

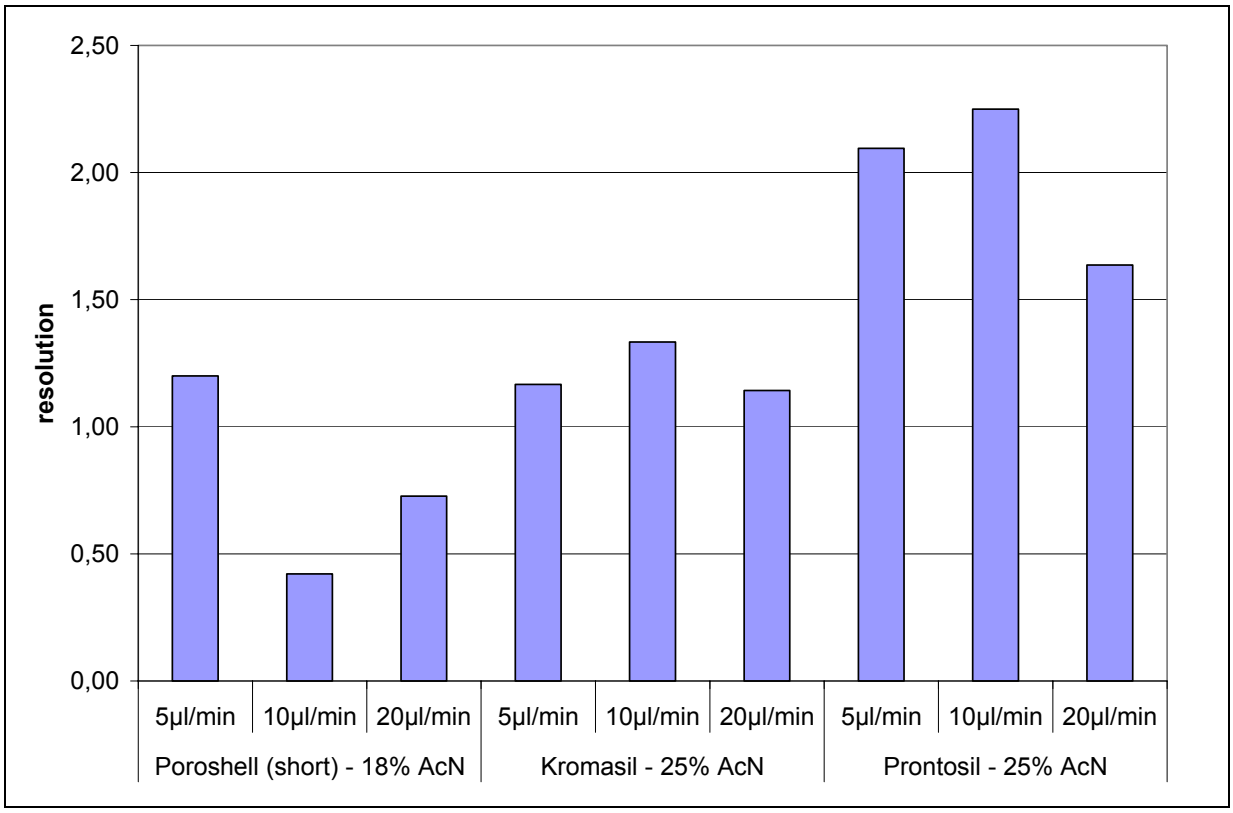

Figure A7: Resolution of the first two peaks at different flow rates.

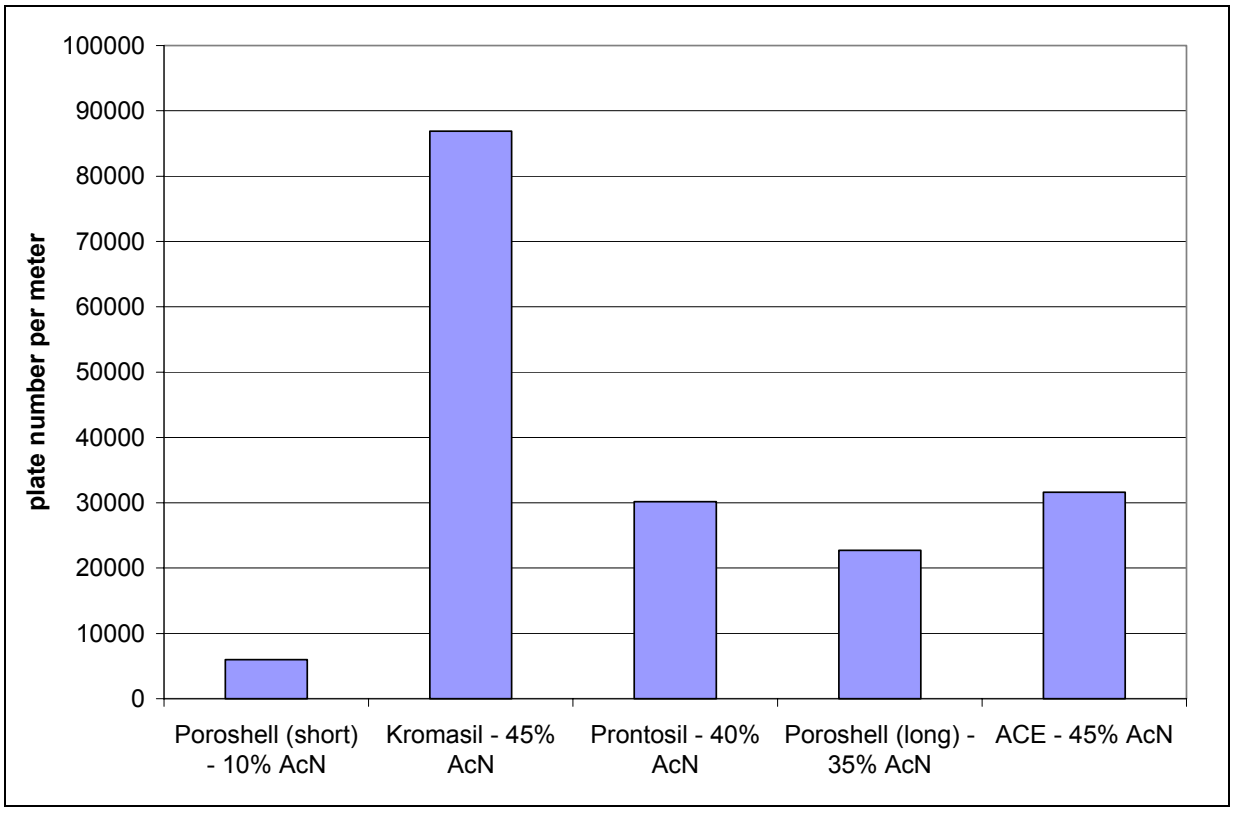

Figure A8: Plate number per meter with toluene as test substance.

The Kromasil column and the ACE column provided the best plate numbers except at high flow rates (figures A3 and A6). High efficiency is desired for the analytical column in a column-switching method. Additionally, they gave the highest k-value for the second peak and thus highest retention (figure A2). High retention is desirable for a precolumn in a column-switching method. Both columns have high efficiency when used for peptides and they have high selectivity (figure A4) and thus high resolution. Additionally, the Kromasil column gave low asymmetry values (figure A5). At high flow rates the Prontosil column is favourable due to no loss in efficiency and high resolution at all flow rates (figures A6-A7).

The Kromasil or the ACE column with a mobile phase containing 23% and 20% acetonitrile respectively at 5 µl/min should be chosen because of the high efficiency provided under these conditions. If efficiency is not that important and high speed is desired, the Prontosil column should be chosen with a mobile phase containing 20% or 23% acetonitrile.

6.1.2 Testing of three different reversed-phase columns as precolumns for focusing of polar analytes

Three different reversed-phase columns were tested for their suitability as precolumns using two different mobile phases (table A2). The test substances that were tried to focus on the columns are given in table A3. A compound could be focused on the column when no breakthrough was observed after the mobile phase had been passed through the column for 30 min.

Conditions used during testing the reversed-phase precolumns are given in table A2. The PS-DVB column was tested with mobile phase A2/B2 only.

Table A2: Conditions used when testing reversed-phase precolumns with H_2O/AcN MP.

Flow rate	5.0 µl/min; 2.0 µl/min*		
Column temperature	Room temperature; 60°C*		
Mobile phases	A1	= 500ml H_2O	+ 500 µl AcOH
	B1 (C_{18})	= 500ml 50%AcN	+ 500 µl AcOH
	B1 (Hypercarb)	= 500ml AcN	+ 500 µl AcOH
	A2	= 500ml H_2O	+ 500 µl HFBA
	B2	= 500ml 50%AcN	+ 500 µl HFBA
Sample	0.1 mg/ml test substance		
Injection volume	50 nl		
Detection	(+) ESI-TOF-MS		

* PS-DVB column

Table A3 gives a summary over all test substances that could be focused on the different reversed-phase precolumns under the conditions described. The numbers in brackets give the minimum percentage AcN that can be present in the mobile phase to get focusing of the whole group analytes.

Table A3: Overview over possible focusing with H_2O/AcN MP.

	C_{18}		*Hypercarb*	
	B1 (AcOH)	*B2 (HFBA)*	*B1 (AcOH)*	*B2 (HFBA)*
Dipeptides				
Gly-Gly	No	No	No	No
Gly-Gly-NH_2	No	No	No	No
Gly-Asp	No	No	No	No
Gly-Glu	No	No	No	No
Gly-His	No	No	No	Yes (0%)
Gly-Tyr	No	No	Yes (0%)	-
Lys-Lys	No	Yes (1%)	Yes (0%)	-
Lys-Phe	No	Yes (1%)	Yes (0%)	-
Ala-Ala	-	-	No	No
Catecholamines				
Dopamine	No	No	No	Yes (0%)
Epinephrine	No	No	No	Yes (0%)
Norepinephrine	No	No	No	Yes (0%)
Serotonin	No	No	Yes (0%)	Yes (0%)
Other compounds				
GABA	No	No	No	No
Acetylcholine	No	No	No	No
Propranolol	Yes (5%)	-	-	-
Peptides				
Leu^5-Enkephalin	Yes (5%)	-	-	-
Met^5-Enkephalin	Yes (5%)	-	-	-
Oxytocin	Yes (5%)	-	-	-
Bradykinin	Yes (5%)	-	-	-
LHRH	Yes (5%)	-	-	-
Neurotensin	Yes (5%)	-	-	-
Angiotensin II	Yes (5%)	-	-	-

6.2 Mass spectrometer conditions

Table A4: Advanced mass spectrometer conditions (printed from MassLynx Tune page).

Source (ES+)		**Advanced**	
Capillary (V)	2800.0	RF DC Offset 1 (V)	10.0
Sample cone (V)	15.0	RF DC Offset 2 (V)	6.0
RF lens (V)	200.0	Aperture (V)	8.0
Extraction cone (V)	3.0	Acceleration (V)	200.0
Desolvation Temp (C)	100.0	Focus (V)	0.0
Source Temp (C)	100.0	Steering (V)	0.0
		MCP Detector (V)	2700.0
Engineer		Pusher Cycle Time	70
Ion Energy (V)	30.0	Pusher Frequency	14285.7143
Tube Lens (V)	5.0		
Grid 2 (V)	65.0	**Instrument configuration**	
TOF Flight tube (V)	4617.0	TDC Gain Control	0.0
Reflectron (V)	1771.0	TDC Edge Control	0.0
Centroid Threshold	1.0	Use 4GHz TDC	YES
Min Points	2.0	Use TTP 4GHz TDC	NO
Np Multiplier	1.00	Source is Z-Spray Mk2	YES
Resolution	4000.0		
Lock Mass	0.000	**Gas Flow**	
Mass Window +/-	0.100	Cone Gas Flow (l/hr)	6
Lteff	1120.3000	Desolvation Gas Flow (l/hr)	251
Veff	4600.0000		
TDC Start (mV)	700.0000	**Vacuum Monitor**	
TDC Stop (mV)	50.0000	Pirani Pressure (mbar)	2.02e0
TDC Threshold	0.0000	Penning Pressure (mbar)	6.01e-7

6.3 Raw data

6.3.1 Retention times and peak areas

Table A5: Retention times of the catecholamines in all injected validation solutions. The values of the first series of the linearity test (TDC stop voltage = 100 mV) are not shown.

Day	*Concentration level*	*Replicate #*	*Retention time [min]*			
			D	*E*	*NE*	*SE*
1	*1*	*1*	4.39	4.17	4.04	5.29
		2	4.20	3.97	3.85	5.29
		3	4.04	3.79	3.63	4.95
	3	*1*	4.32	4.09	3.95	5.09
		2	4.07	3.84	3.67	4.79
		3	4.12	3.85	3.67	4.82
	5	*1*	4.42	4.17	4.07	5.25
		2	4.34	4.10	3.99	5.12
		3	4.34	4.10	3.97	5.10
2	*1*	*1*	4.37	4.12	4.02	5.20
		2	4.35	4.14	4.04	5.02
		3	4.22	4.00	3.94	5.20
	3	*1*	4.09	3.85	3.72	4.94
		2	4.24	4.00	3.87	5.04
		3	4.30	4.07	3.94	5.09
	5	*1*	4.37	4.15	4.00	5.15
		2	4.35	4.12	3.99	5.09
		3	4.34	4.14	3.95	5.07
3	*1*	*1*	4.35	4.14	4.02	4.80
		2	4.27	4.07	3.95	5.29
		3	4.27	4.05	3.92	5.17
	3	*1*	4.29	4.07	3.92	5.09
		2	4.30	4.07	3.92	5.05
		3	4.29	4.05	3.92	5.04
	5	*1*	4.27	4.05	3.90	4.95
		2	4.27	4.05	3.90	4.95
		3	4.25	4.02	3.85	4.95

Table A6: Peak areas of the catecholamines in all injected validation solutions.

Day	*Concentration level*	*Replicate #*	*Peak area*			
			D	*E*	*NE*	*SE*
1°	*1*	*1*	17	18	11	0
		2	23	20	11	3
		3	23	27	16	7
	2	*1*	50	34	33	14
	3	*1*	98	78	78	25
		2	100	71	71	27
		3	108	76	70	32
	4	*1*	166	188	138	64
	5	*1*	277	331	161	116
		2	288	323	146	119
		3	287	295	114	120
	6	*1*	405	441	234	380
1	*1*	*1*	44	31*	56	10*
		2	47	30*	55	29*
		3	50	29*	46	42*
	3	*1*	262	133	114	102
		2	255	125	111	104
		3	278	182	151	126
	5	*1*	484	655	411	130
		2	593	706	408	218
		3	582	665	380	240
2	*1*	*1*	45	22*	42	14*
		2	41	24*	37	8*
		3	46	23*	42	14*
	3	*1*	218	181	135	87
		2	241	187	133	94
		3	266	187	142	110
	5	*1*	573	684	291	261
		2	583	672	273	261
		3	578	620	246	266
3	*1*	*1*	36	22*	38	12*
		2	36	20*	42	11*
		3	47	25*	44	26*
	3	*1*	245	157	125	118
		2	270	161	133	142
		3	256	159	121	127
	5	*1*	534	586	241	317
		2	444	540	218	318
		3	446	534	214	319

°First series of linearity test (TDC stop voltage = 100 mV). Generally, all values were integrated permitting smoothing of the peaks. The values marked with * were obtained without smoothing the peak. This was done because it was difficult to determine the peaks with smoothing.

Table A7: Peak areas of E and E with oxidation taken into account at the 5th concentration level; percentage increase of the peak area.

Day	*Replicate*	*Peak area*		*% increase*
		Epinephrine	*Epinephrine + oxidation*	
Day 1	*1*	655	705	7.6
	2	706	740	4.8
	3	665	730	9.8
Day 2	*1*	684	702	2.6
	2	672	714	6.3
	3	620	640	3.2
Day 3	*1*	591	616	4.2
	2	540	571	5.7
	3	534	571	6.9

6.3.2 Between-day linearity test

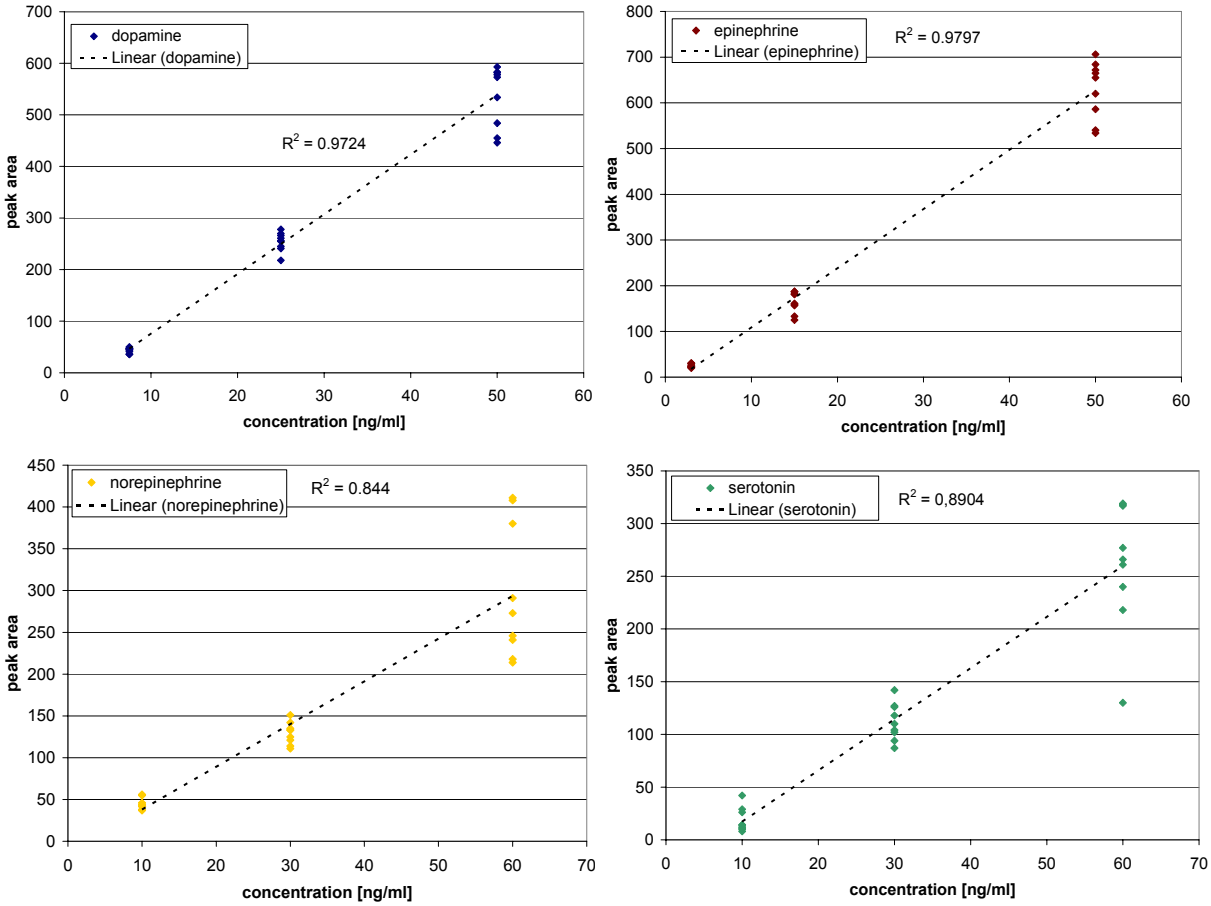

Figure A9: Between-day linearity test.

6.3.3 Statistics

Generally, statistics have to be used with care. Especially the use of the test for outliers demands rational deal with the numbers obtained. In the field of medical science unusual high or low values can occur due to abnormal high or low levels of the analyte in a real sample.

In this work, no real samples were used, so the test can be used without the danger of changing the interpretation of the results.

6.3.3.1 Test for outliers

To test if one suspect value can be treated as an outlier, a Grubb's test was performed. This test is recommended by ISO in preference to Dixon's test (Q-test) [47]. The suspected value passed the Grubbs test and was thus kept, but it was close to be rejected.

$$G = \frac{\left| suspected\ \ value - \bar{x} \right|}{s} \tag{A1}$$

6.3.3.2 Analysis of variance (ANOVA)

The analysis of variance was performed as described in [47]. ANOVA tests whether the difference between the sample means is too great to be explained by random errors. The number of samples is in this case the number of days; the number of members is in this case the number of replicates. ANOVA was performed using a Casio Model CFX-9850GB Plus calculator. It calculates the within-day and between-day variances. The relative standard deviations for within-day and between-day were calculated by (A2) and (A3) respectively, where MS is the factor mean of squares (mean of squares variation between groups = between-day variance), MSe is the error mean of squares (mean of squares variation within groups = within-day variance) and $\bar{x}$ is the overall mean.

$$RSD\ [\%] = \frac{\sqrt{MS} \cdot 100}{\bar{x}} \tag{A2}$$

$$RSD\ [\%] = \frac{\sqrt{MSe} \cdot 100}{\bar{x}} \tag{A3}$$

6.3.3.3 Within-day precision

The within-day precision calculated in the conventional way, e.g. by calculating the relative standard deviation (RSD) from the retention times and the peak areas using Microsoft Excel is given in table A8 and A9.

Table A8: Within-day precision of the retention times using a TDC stop voltage of 50 mV.

		Dopamine RSD [%]	*Epinephrine RSD [%]*	*Norepinephrine RSD [%]*	*Serotonin RSD [%]*
c level #1	*day 1*	0.2	0.2	0.2	0.2
	day 2	0.08	0.08	0.05	0.1
	day 3	0.05	0.05	0.05	0.3
c level #3	*day 1*	0.1	0.1	0.2	0.2
	day 2	0.1	0.1	0.1	0.08
	day 3	0.01	0.01	0	0.03
c level #5	*day 1*	0.05	0.04	0.05	0.08
	day 2	0.02	0.02	0.03	0.04
	day 3	0.01	0.02	0.03	0

Table A9: Within-day precision of the peak areas using a TDC stop voltage of 50 mV.

		Dopamine RSD [%]	*Epinephrine RSD [%]*	*Norepinephrine RSD [%]*	*Serotonin RSD [%]*
c level #1	*day 1*	6.4	3.3	11	60
	day 2	4.5	21	18	12
	day 3	11	4.0	4.2	33
c level #3	*day 1*	6	4.3	7.2	29
	day 2	10	1.9	3.5	12
	day 3	0.9	5.2	8.4	3.1
c level #5	*day 1*	16	11	7.4	51*
	day 2	4.9	1.3	4.8	9.4
	day 3	10	5.1	6.5	0.3

* A value that is near to be rejected by Grubbs test is included.

6.4 The neuron and signal submission by neurotransmitters

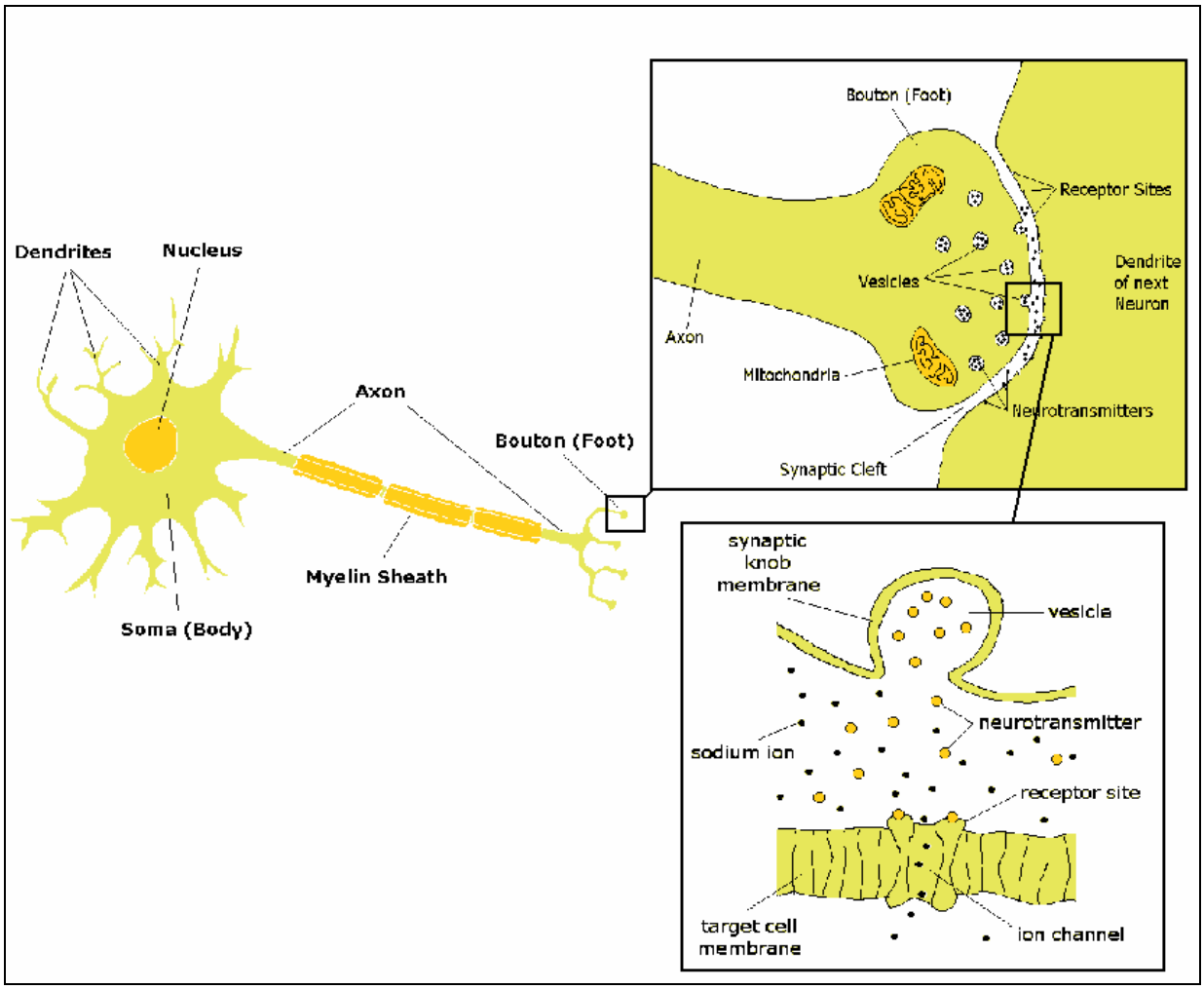

Figure A10: The neuron with the synapse (enlarged) and the receptor site (enlarged). (http://www.ship.edu/~cgboeree/theneuron.html)